Growth Characteristics of Free Tumor Cells Transferred Serially in the Peritoneal Fluid of the Mouse*

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It is common knowledge that the presence of abdominal tumors in cancer patients and the growth of peritoneal implants in laboratory animals may be associated with the appearance of free tumor cells in the peritoneal fluid (2–30). However, the specific biological characteristics of these free cells —their growth cycle, growth potential, and relationship to peritoneal implants in the same animal —have not been experimentally investigated. In the present paper, the number of cells and their growth rate in the peritoneal fluid, as well as their regressive changes, were estimated repeatedly in the same mouse, which received intraperitoneal inoculation of a predetermined number of tumor cells. By carrying out serial intraperitoneal transfers of Sarcoma 37 cells and of malignant lymphoma cells from mouse to mouse, at intervals of 3–7 days, it was possible to maintain continuous multiplication of these cells in the peritoneal fluid for more than 10 months. Our data indicate the possibility of using mouse tumor cells in in vivo culture for the quantitative study of various problems in the field of research.

MATERIAL AND METHODS

Two tumors were chosen for this study: (a) S-37; this tumor is not strain specific; it grows readily in different strains of mice, with varying percentages of regression, and elicits a marked leukocytic reaction in the peritoneal fluid; (b) a malignant lymphoma arising spontaneously in the thymus of a strain dba (212) mouse. The tumor was found to be practically strain specific, growing

poorly if at all in strains other than dba (212). In strain dba (212) it grows steadily as a localized tumor with almost no regression. It was found in preliminary experiments to elicit a relatively mild leukocytic reaction. The mice used in quantitative experiments with S-37 were all males of the Carworth Farms W Strain (CFW), but dba, Swiss, and C3H mice also were found convenient in preliminary studies. Male dba (212) mice were used in the work with malignant lymphoma.

Subcutaneous implants 6 or 7 days old were ground in a tissue press and suspended in a convenient volume of 0.85 per cent NaCl solution. Before inoculation of the requisite number of tumor mash cells, the number of viable cells in 0.1 cc. of the mash suspension was determined. The suspension was separated from tissue fragments and cell clumps by sedimentation and decantation. A sample of suspension was placed in a hemocytometer previously coated with a 0.02 per cent alcoholic solution of neutral red. Five minutes later a count was made to compute the number of viable tumor cells per 0.1 cubic centimeter. Cells with nuclei showing any trace of neutral red stain were considered dead. This count indicated the amount of dilution necessary to obtain the requisite number of viable tumor cells per cubic centimeter. Tumor cell counts in the peritoneal exudate were made in the same way, except that the total number of viable tumor cells in 0.1 cubic centimeter of original exudate diluted 1:10 or 1:20 was computed by combined methods of total cell counts in the hemocytometer and differential tumor cell counts in stained smears. One smear was stained with Wright's stain and the other by the acetoorcein method. The results of counts in both

smears usually agreed within 5 per cent. Information from these counts was used to determine the number of viable tumor cells, the percentage of mitoses, and the percentage of necrotic tumor cells per 0.1 cubic centimeter of the original exudate. Peritoneal exudate was removed by exploratory puncture with glass capillary pipettes. Preliminary experiments have demonstrated that the cellular composition of the peritoneal fluid does not vary significantly in samples withdrawn from different pockets of the peritoneal cavity in the same mouse.

RESULTS

TUMOR CELLS IN PERITONEAL FLUID

Counts on specimens of the peritoneal fluid obtained from twelve normal CFW mice indicated a wide range in the number of cells—from 63,000 to 160,000 per cubic millimeter. In the majority the lymphoid cells were most numerous. In differential counts the average percentages found were as follows: lymphocytes, 60.4–80.5 per cent; polymorphonuclears, 0–0.7 per cent; large monocytes, 7.5–29.2 per cent; macrophages, 4.0–23 per cent; and mast cells, 1.0–4.6 per cent (Fig. 1). Similar counts were found in the peritoneal fluid of fifteen dba mice. The fate of normal mouse tissue cells in the peritoneal fluid was investigated in four groups each of fifteen mice of various strains (5 C3H, 5 dba, and 5 CFW) that received intraperitoneal injections of various doses (1 cc. of 1:10 to 1:500 dilution of tissue mash) of mashed liver, lung, spleen, and brain from normal mice of the same strain. Only detritus of these cells was found in all specimens of peritoneal fluid withdrawn 4 or 5 days after injection. No mitotic figures were found at any time in the injected cells withdrawn with the peritoneal fluid. Thus, the cells of the normal tissues tested failed to multiply or even to survive in the peritoneal fluid. These preliminary experiments served as control experiments for the following studies on tumor cells.

A suspension of mashed S-37 tumor cells (Fig. 2) obtained from a 6-day-old subcutaneous transplant was prepared and diluted to the concentration of 10,000 cells per 0.1 cubic centimeter. Doses of 10,000 cells were injected intraperitoneally into each of 27 mice, and samples of the peritoneal fluid were taken at various intervals for total and differential cell counts. In fourteen mice the exploratory punctures of the abdomen were repeated at short intervals (after 6, 24, 48 hours, etc.) over a period of 7 days, and in thirteen mice at longer intervals (every fourth or fifth day) over a period of 12 days. The results are presented graphically in Chart 1. These results indicated that the number of sar-

coma cells and the percentage of mitotic figures decreased during the first 24 hours after inoculation. This decrease (lag period) was followed by a slow increase during the next 24 hours (24–48 hours after inoculation), and in the 48–72-hour period there was a sharp increase both in the number of cells and in the percentage of their mitoses. After this initial stage of rapid multiplication, there was a more slow but steady increase in the number of cells and in the percentage of their mitotic figures for a period of 4–5 days. Finally, a sharp rise in the relative number of necrotic tumor cells was noted and at the same time a steady decrease in the total number of sarcoma cells per 0.1
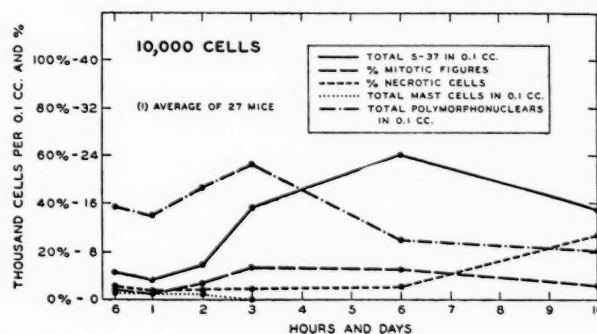

CHART 1.—Growth cycle in the peritoneal fluid of 10,000 S-37 cells from a subcutaneous implant.

cubic centimeter. Chart 1 also shows that there is an early rise in the curve for the number of polymorphonuclear leukocytes in the peritoneal fluid which reaches a peak on the third day after inoculation. It is of interest that on the tenth day after inoculation, when the percentage of necrotic cells has increased significantly, the curve for polymorphonuclear leukocytes continues to fall. For the first 24 hours after inoculation, when there was an apparent decrease in the number both of tumor cells and of mitotic figures, the majority of the cells appeared shrunken to the size of a small lymphocyte (Fig. 3). Between 24 and 48 hours after inoculation, the tumor cells somewhat increased in size (Fig. 4). By 48–72 hours, at the beginning of initial rapid multiplication, the average size returned to that characteristic of healthy S-37 cells (Figs. 5 and 6). Figures 7 and 8 illustrate the appearance of specimens taken for differential counts at the time of maximum cell growth and at the time of terminal necrosis and death of tumor cells, respectively, for the series inoculated with 10,000 cells.

Similar studies were made using smaller numbers of S-37 cells in the original inoculum. With 1,000 cells and, more obviously, with 100 cells inoculated, the peak of the curves for the number

of tumor cells and for the relative number of mitoses occurred later and did not reach as high levels as when 10,000 cells were inoculated. The curves for necrotic cells, on the other hand, rose earlier and reached a higher peak.

The curves of growth of similar numbers of malignant lymphoma cells (Charts 2 and 3) contrast strikingly with the curves of S-37; they show continuous increase in the number of tumor cells
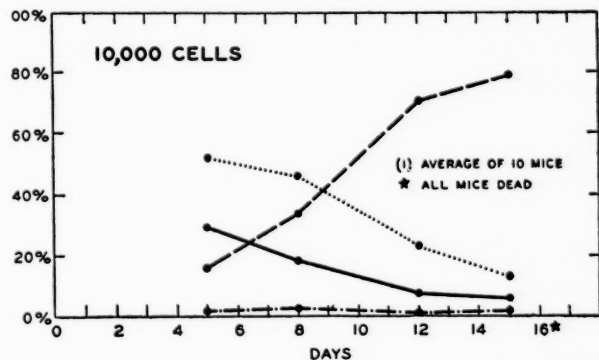

CHART 2.—Growth in the peritoneal fluid of 10,000 malignant lymphoma cells from a subcutaneous implant (see legend of Chart 3).
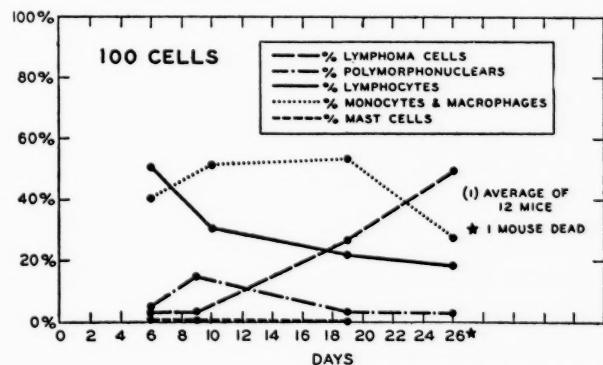

CHART 3.—Changes in the cellular composition of the peritoneal fluid following intraperitoneal inoculation of 100 malignant lymphoma cells from mashed subcutaneous implants.

until the death of the animal and at the same time continuous decrease in the number of all types of leukocytes. The curves of monocytes remain, as a rule, above the curves of lymphocytes, while the curve of polymorphonuclears begins at a much lower level and only slightly above the curve of mast cells. The curves of lymphoma cells rise more abruptly, and the concentration of these cells increases at a higher rate after inoculation of a larger number of cells. Briefly, the changes in the cellular composition consist in "overgrowing" of leukocytes by multiplying lymphoma cells which show the tendency to approach the condition of a "pure culture" in the peritoneal fluid. These results are

illustrated by stained smears showing the cellular composition of inoculated material (mashed subcutaneous implant) and of the peritoneal fluid in inoculated animals before inoculation (in a normal dba [212] mouse) and at consecutive growth stages after inoculation (Figs. 9–14).

MULTIPLICATION OF TUMOR CELLS IN THE PERITONEAL FLUID

Abdominal massage of mice bearing peritoneal implants of Sarcoma 37.—The possibility existed that the increase in the number of free sarcoma cells in the peritoneal fluid might result from the disintegration or sloughing of peritoneal implants induced by injection of tumor mash cells. To test this possibility, ten mice received intraperitoneal inoculations, each with approximately 1,000 sarcoma cells from the peritoneal fluid. Nine to 12 days after inoculation, a small amount of peritoneal fluid was withdrawn from each mouse. The number of sarcoma cells and of leukocytes in each specimen was counted. Immediately afterward, the abdomen of each animal was energetically massaged for 3 minutes. A small amount of fluid was again withdrawn and examined as before. At the autopsy performed on the thirteenth day after inoculation, peritoneal tumors were found in the abdominal peritoneum of nine out of ten animals (one without tumor). It appeared that in two out of ten mice the percentage of sarcoma cells was increased after the massage by 7 per cent and in only one mouse by 23 per cent. In other mice it was approximately unchanged or even decreased. In some instances, there was an increase in number of lymphocytes in the peritoneal fluid after the massage. Thus, even a severe mechanical disturbance did not induce any consistent disruption or sloughing of small peritoneal tumors.

Multiplication during rapid serial transfers.—Since Algire and Chalkley (1) have shown that inoculated tumor tissue elicited signs of tumor vascularization (capillary growth) as early as the fourth day after inoculation, we have carried out successive transfers of peritoneal fluid containing S-37 cells at intervals of 2 and 3 days. Mice received intraperitoneal inoculations of known numbers of sarcoma cells (10,000 in the first series and 1,000 in the second series). The peritoneal fluid of inoculated mice was transferred, after 3 days, into new mice; from these mice, after 2 or 3 days, into new mice, etc.

Altogether, seven successive transfers were carried out in seven groups, each of five mice, at the same intervals. Three days after the last transfer a small amount of peritoneal fluid was withdrawn from each ultimate recipient in each series, and

the number of sarcoma cells in each specimen was counted and computed per 0.1 cubic centimeter. All animals examined contained more than 0.1 cubic centimeter of fluid, but even in 0.1 cubic centimeter the number of sarcoma cells was higher than their number in the inoculum received by the first group of mice in the series. These results indicate that tumor cells increase in number in the peritoneal cavity during serial transfers of the peritoneal fluid from mouse to mouse in the absence of vascularized tumor growth.

For larger series, transfers from mouse to mouse were made alternately at 3- and 4-day intervals. Each series was initiated by intraperitoneal injection of 50,000 to 250,000 sarcoma cells, from mashed subcutaneous S-37 implants, into eight mice. Their peritoneal fluid was withdrawn after 4 days and examined. The specimen showing the highest number of sarcoma cells and of their mitoses was used for the transfer of peritoneal fluid into eight new mice, in doses of 0.1–0.25 cubic centimeter. After an interval of 3 days the peritoneal fluid of these mice was examined, and a suitable specimen was selected for use in the second transfer into a new group of six mice. This specimen contained, in 0.1 cubic centimeter, 20,000–30,000 sarcoma cells, 15 or 20 per cent of which showed mitotic figures. The "recipients" of the second transfer were used as "donors" of the fluid for the third transfer, after 4 days. This procedure was repeated regularly for 10 months. The descendants of cells issued from two different subcutaneous implants were propagated for 64 transfers as three strains of free sarcoma cells (D, F, and G) in three separate series of mice. After the first 15–20 transfers of each strain, the peritoneal fluid of most animals contained at each transfer a large number of sarcoma cells (50,000–70,000 per 0.1 cubic centimeter) with a high percentage of mitotic figures (25 per cent or more), and the selection of the "best" donor was no longer necessary. The fluid was characterized by its uniform grayish opacity, the absence of macroscopic cell clumps and blood, and by its albuminoid consistency. After each transfer intraperitoneal inoculation of the sarcoma cells provoked a rapid accumulation of an effusion in the peritoneal cavity, and the cells continued to multiply in this medium.

Similar transfers of peritoneal fluid containing malignant lymphoma cells were carried out serially from mouse to mouse at weekly intervals, until it was noticed that, after the sixteenth to nineteenth transfer, three or four out of ten mice at each transfer died within a few days. Therefore, at the twentieth transfer, the withdrawn peritoneal fluid was diluted 1:5 and transferred in doses of 0.5

cubic centimeter of this dilution. All inoculated mice survived for at least a week. After the 24th transfer the dose of transferred fluid was cut again, for the same reason, to 0.5 cubic centimeter of a 1:26 dilution of the peritoneal fluid; after the 26th transfer, to 0.5 cubic centimeter of a 1:52 dilution; at the 33d transfer, to 0.25 cubic centimeter of a 1:52 dilution; and on the 40th transfer, to 0.1 cubic centimeter of the same dilution.

Subcutaneous "Auto-inoculation"

In nearly all mice that received intraperitoneal inoculations of 10,000 to 1,000 sarcoma cells, and in some mice inoculated with 100 cells, a nodule, sometimes growing into a tumor, sometimes regressing, developed subcutaneously at the site of intraperitoneal injection. This phenomenon of subcutaneous auto-inoculation of the mouse by tumor cells from the peritoneal cavity seems to be related to the initial rate of multiplication of sarcoma cells inoculated into the peritoneal fluid (Table 1). There was some question as to the origin of the tumor cells which gave rise to the subcutaneous nodules following intraperitoneal inoculation—whether they were introduced originally in a subcutaneous position as a result of leakage from the syringe on injection or whether they migrated into the wound after a period of growth in the peritoneal cavity. In an attempt to settle this question, twenty animals each received three widely spaced abdominal punctures with a sterile injection needle, followed by a single intraperitoneal injection of S-37 cells at still another site. Nodules later developed at the site of all four punctures in some cases, and in others nodules developed at several sites of puncture but not at the site of tumor cell inoculation, indicating that S-37 cells migrated into the punctured areas from the peritoneal fluid.

Growth Cycle in Serial Intraperitoneal Transfers of Tumors

Continuity of multiplication of injected cells.—Doses of 10,000 sarcoma cells from the peritoneal fluid were inoculated intraperitoneally into 31 mice. In 14 mice the exploratory punctures of the abdomen were repeated at short intervals (after 6, 24, 48 hours, etc.) during 7 days and in 17 mice at longer intervals (every fourth or fifth day) during 11 days. The withdrawn specimens of fluid were examined for total and differential counts of sarcoma cells and leukocytes. Since the results in all groups showed complete agreement, they were pooled and plotted as a chart (Chart 4). A similar series of changes in the number, activity, and viability of S-37 cells from the same source was studied by repeated cell counts when smaller numbers of these

cells were transferred intraperitoneally (Chart 5).

These data show that the inoculation was followed by a short lag period of approximately 24 hours, during which time the number of sarcoma cells remained on about the same level (or even decreased in a few mice), while the percentage of mitotic figures sharply decreased. The curves of sarcoma cells and of their mitoses rose sharply between the 24th and 48th hours, indicating that the viability of inoculated cells was not impaired by transfers. There was no shrinkage of transferred cells. On the contrary, their size was often markedly increased during the first 24 hours (Figs. 15–17). The maximum percentage of mi-

totic figures was reached on the fourth day, while the increase in the total number of cells continued until the sixth day, when it began to decrease. The curve of necrotic cells looked like an inverted curve of cell multiplication, thus illustrating the parallelism between the loss of activity and the loss of viability in proliferated cells. Figures 18–20 illustrate the appearance of specimens of these cells taken on the third, the sixth, and the tenth days after the transfer.

Similar experiments with malignant lymphoma cells from serial intraperitoneal transfers are illustrated by Figures 21 and 22 and by Charts 6 and 7.

The growth characteristics of mash cells and of

TABLE 1

GROWTH OF SUBCUTANEOUS TUMORS IN THE ABDOMINAL WALL AT THE SITE OF INTRAPERITONEAL INOCULATION OF S-37 CELLS

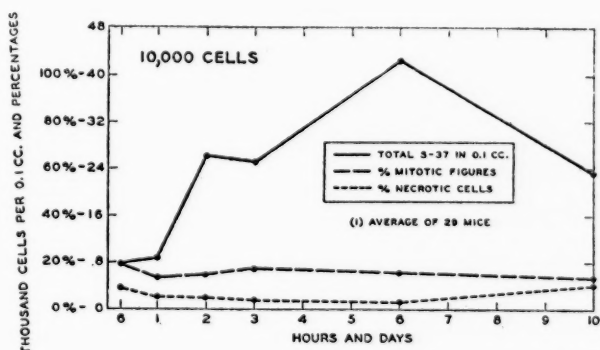| NO. OF CELLS INOCULATED	No. OF MICE INOCULATED	SOURCE OF MATERIAL	No. OF MICE SHOWING AUTO-INOCULATION AT THE SITE OF INJECTION OR PUNCTURE			No. OF DAYS BETWEEN THE DATE OF INOCULATION AND DATE OF APPEARANCE OF GROWTH		AV. PER CENT MITOSES 3–6 DAYS AFTER INOCULATION
			No growth	Nodule only	Nodule, later tumor	Nodule	Tumor	
10,000	15	Tumor mash	0/15	3/15	12/15	6	6	10.2
1,000	14	"	1/14	3/14	10/14	12	19	4.8
100	14	"	4/14	3/14	7/14	18	22	2.7
10,000	17	Peritoneal fluid	0/17	1/17	16/17	6	6	14.8
1,000	14	"	0/14	1/14	13/14	8	12	10.1
100	14	"	2/14	4/14	8/14	14	20	2.8

CHART 4.—Growth cycle in the peritoneal fluid of 10,000 S-37 cells from intraperitoneal serial transfers.

CHART 6.—Changes in the cellular composition of the peritoneal fluid following intraperitoneal inoculation of 10,000 lymphoma cells from the serial intraperitoneal transfers.
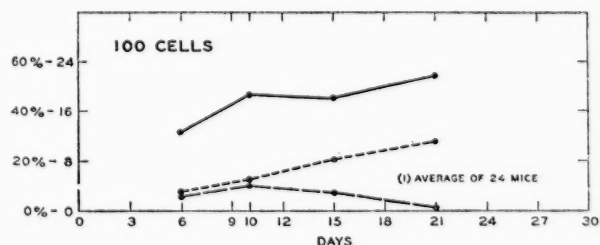

CHART 5.—Growth cycle in the peritoneal fluid of 100 S-37 cells from intraperitoneal serial transfers (see legend of Chart 4).
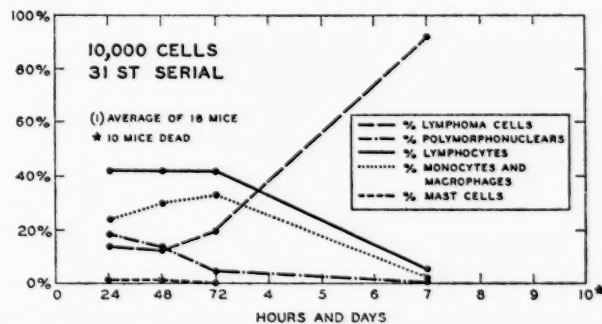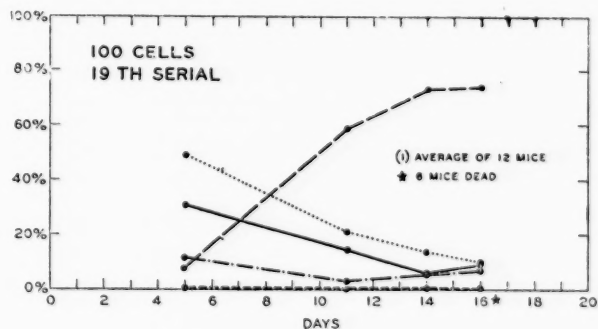

CHART 7.—Growth cycle in the peritoneal fluid of 100 malignant lymphoma cells from the serial intraperitoneal transfers (see legend of Chart 6).

cells from peritoneal fluid were compared as to the levels of growth and of necrosis at certain intervals after intraperitoneal inoculation of various doses (10,000, 1,000, and 100) of cells. This comparison suggested that for each group of mice inoculated with the same number of tumor cells, the level of cell multiplication was higher and the rate of cell death lower for descendants of cells from peritoneal fluid than for descendants of mash cells (Tables 2 and 3).

Growth potential of tumor cells cultured in the peritoneal fluid.—In order to investigate the significance of this observation more completely, a series of experiments with S-37 in CFW mice and with lymphoma in dba mice was carried out in which different numbers of cells were inoculated (10,000, 5,000, 1,000, 100, and 10). They were obtained either from mash tumor or from peritoneal fluid and were injected either subcutaneously or intraperitoneally. The number of mice in each group varied from 8 to 25. After the inoculations were made, the mice were examined at intervals over a period of 18 days for the appearance of tumor nodules. Notes were taken as to the time of appearance of nodules and of their changes in size. The results were clear cut and demonstrated that, when injected subcutaneously, tumor cells from the peritoneal fluid produced larger tumor nodules more quickly than were produced by the same number of cells from mashed subcutaneous

TABLE 2

COMPARISON OF THE GROWTH CHARACTERISTICS OF SARCOMA 37 CELLS FROM MASHED SUBCUTANEOUS IMPLANTS AND FROM PERITONEAL FLUID*

AVERAGE RESULTS AT VARIOUS INTERVALS AFTER INOCULATION		SOURCE OF SARCOMA CELLS; NUMBER OF MICE IN EACH GROUP					
		from mashed implants			from peritoneal fluid		
		5,000 cells 10 mice	1,000 cells 14 mice	100 cells 14 mice	5,000 cells 10 mice	1,000 cells 24 mice	100 cells 24 mice
First count:	Number of sarcoma cells (in 0.1 cc.)	11,700	7,000	7,100	14,000	17,600	8,900
	Per cent of mitoses	9.7	4.8	2.7	20.3	10.1	2.8
	Per cent of necrotic cells	3.2	5.7	2.8	3.7	4.2	5.4
Second count:	Number of sarcoma cells (in 0.1 cc.)	30,100	15,800	21,900	57,100	32,200	15,700
	Per cent of mitoses	8.2	13.4	6.6	12.6	19.9	12.4
	Per cent of necrotic cells	6.3	8.3	10.6	3.1	3.0	8.2
Third count:	Number of sarcoma cells (in 0.1 cc.)	10,700	16,700	21,100	47,500	41,200	23,000
	Per cent of mitoses	1.7	12.8	3.0	5.8	21.1	12.3
	Per cent of necrotic cells	68.8	11.1	57.0	12.7	8.3	15.2
Fourth count:	Number of sarcoma cells (in 0.1 cc.)	All mice dead	Not counted		All mice dead	Not counted	
	Per cent of mitoses		2.9	4.3		0.75	21.1
	Per cent of necrotic cells		6.1	92.0		1.8	77.5

*Similar data for mice inoculated with 10,000 cells from mashed implants and peritoneal fluid are shown on Charts 1 and 4.
In mice inoculated with 5,000 cells, the first count was performed after 3 or 4 days, the second after 7–8 days, the third after 10 or 11 days. In mice inoculated with 1,000 cells, the first count was made after 4–5 days, the second after 8–9 days, the third after 12–13 days, and the fourth after 16–18 days; in mice inoculated with 100 cells, the first count after 6–7 days, the second after 10–14 days, the third after 15–20 days, and the fourth after 27 days.

TABLE 3

COMPARISON OF THE GROWTH CHARACTERISTICS OF MALIGNANT LYMPHOMA CELLS FROM MASHED SUBCUTANEOUS IMPLANTS AND FROM PERITONEAL FLUID*

AV. RESULTS AT VARIOUS INTERVALS AFTER INOCULATION		SOURCE AND NUMBER OF LYMPHOMA CELLS; NUMBER OF MICE IN EACH GROUP					
		from mashed implants			from peritoneal fluid		
		10,000 cells 10 mice	1,000 cells 10 mice	100 cells 10 mice	10,000 cells 12 mice	1,000 cells 12 mice	100 cells 12 mice
First count:	Number of lymphoma cells (in 0.1 cc.)	6,800	3,800	7,100	51,800	19,300	4,300
	Per cent of mitoses	4.5	2.1	2.5	8.7	7.5	8.5
	Per cent of necrotic cells	7.5	13.0	8.0	9.5	5.4	5.5
Second count:	Number of lymphoma cells (in 0.1 cc.)	12,000	24,100	16,200	132,000	85,800	73,700
	Per cent of mitoses	2.5	7.0	7.5	15.3	5.3	11.4
	Per cent of necrotic cells	7.0	10.0	15.0	6.0	3.3	5.5
Third count:	Number of lymphoma cells (in 0.1 cc.)	121,000	103,000	25,000	113,000	132,000	128,000
	Per cent of mitoses	16.0	11.7	12.1	6.7	12.0	8.0
	Per cent of necrotic cells	14.0	15.0	23.1	12.0	12.6	11.0
Growth of subcut. tumors at site of inoc.		0/10	5/5	10/0	12/0	12/0	11/1

* In mice inoculated with 10,000 cells, the 1st count was performed after 4 days, the 2d count after 7–9 days, and the 3d count after 10–11 days. In mice inoculated with 1,000 cells, the 1st count was made after 5–7 days, the 2d count after 10–11 days, and the 3d after 13–15 days. In mice inoculated with 100 cells, the 1st count was made after 9–10 days, the 2d after 11 days after per. fl. inoculation and 19 days after mash inoculation, the 3d count after 14 and 25 days, respectively.

tumors. They also showed that more tumor nodules developed by "auto-inoculation" following intraperitoneal inoculation than following subcutaneous inoculation.

DISCUSSION

Our results have shown that continued multiplication of tumor cells can be obtained by serial intraperitoneal transfers of these cells from mouse to mouse, at intervals of 3–7 days. This method of tumor cell culture in the peritoneal fluid *in vivo* enabled us to study the growth cycle of tumor cells on a quantitative basis. This method also provided data concerning the quantitative relationship between the number of cells inoculated either subcutaneously or intraperitoneally and the rate of growth of resulting subcutaneous implants. Finally, the intraperitoneal transfer of free tumor cells from mouse to mouse enabled us to obtain subcutaneous tumors by intraperitoneal inoculation of very small numbers (100 or 10) of tumor cells from the peritoneal fluid.

The results also show that tumor cells inoculated into the peritoneal cavity obey the same quantitative rules as cultures of free organisms—i.e., the amount and rate of cell multiplication are proportional to the size of the inoculum. However, they also demonstrate that when small numbers of cells are inoculated such cells appear to be handicapped in their proliferation. They show lower percentages of mitosis and higher percentages of necrotic cells than do descendants of larger inocula in corresponding stages of growth.

We feel that the material presented in this study serves as an adequate demonstration that quantitative as well as qualitative data may be obtained from an analysis of free tumor cells growing in the peritoneal fluid of mice. It is hoped that the data may serve as a basis for the development of an additional screening method to test the activity of physical and chemical agents against the growth of tumor cells, a method which would supplement the various screening technics at present in use.

SUMMARY AND CONCLUSIONS

1. Requisite numbers of Sarcoma 37 or malignant lymphoma cells, obtained either from subcutaneous implants or by serial transfers in the peritoneal fluid, were inoculated intraperitoneally into several groups of CFW or dba mice. The rate and the amount of cell growth in each mouse were studied quantitatively. Specimens of peritoneal fluid were withdrawn repeatedly, at various time intervals, from the same mouse, for total and differential cell counts (number of tumor cells per 0.1

cubic centimeter, percentage of mitoses, of necrotic elements, and of leukocytes). The results from each group of mice were tabulated and plotted in graphs.

2. The growth in the peritoneal fluid of tumor cells from both strains presented the following essential features: (*a*) Intraperitoneally inoculated tumor cells multiplied in the peritoneal fluid independently of peritoneal implants, i.e., before and sometimes without the growth of tumor tissue in the peritoneum; thus, the multiplication of free tumor cells appeared to be a primary phenomenon. (*b*) Free tumor cells multiplied continuously in the peritoneal fluid in serial intraperitoneal transfer of this fluid from mouse to mouse, thus appearing as a culture of the inoculated tumor strain. (*c*) Serial intraperitoneal transfers increased the growth potential of tumor cells in the peritoneal fluid and in the subcutaneous tissue. (*d*) For the cells of the same source, i.e., of the same growth potency, the amount and the rate of growth were proportionate to the number of inoculated cells. It is suggested that these phenomena are probably not specific for the tumor strains used in our work, but may be reproduced with other tumor strains.

3. The growth of free malignant lymphoma cells in the peritoneal fluid, as compared with the growth of S-37 under the same conditions of experiment, presented the following special features: (*a*) The growth cycle of lymphoma cells in the peritoneal fluid lacked the final stage of inactivity and of partial regression which terminated the growth cycle of S-37 free cells. (*b*) The percentage of lymphoma cells in the cellular content of the peritoneal fluid increased in each mouse steadily from the end of the lag period until the death of the animal. This phenomenon contrasts sharply with the intense leukocytic reaction to S-37 cell multiplication in CFW mice. These differences in growth characteristics of free lymphoma cells in the peritoneal fluid would appear to be attributable to the essential difference between the "strain specific" quality of lymphoma cells for dba mice and the "foreign" nature of S-37 cells for CFW mice.

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FIGURES 1–8.—Appearance of S-37 cells from subcutaneous implants in various stages of their growth cycle in the peritoneal fluid.

FIG. 1.—Peritoneal fluid from a CFW mouse before inoculation. Wright, ×800.

FIG. 2.—S-37 Tumor mash used for inoculation. Aceto-orcein, ×800.

FIG. 3.—Appearance of inoculated S-37 cells in the peritoneal fluid 6 hours after inoculation of 10,000 cells. Aceto-orcein, ×800.

FIG. 4.—Appearance of inoculated S-37 cells in the peritoneal fluid after 24 hours. Aceto-orcein, ×800.

FIG. 5.—Appearance of inoculated S-37 cells in the peritoneal fluid after 48 hours. Aceto-orcein, ×800.

FIG. 6.—Appearance of inoculated S-37 cells in the peritoneal fluid after 72 hours. Aceto-orcein, ×800.

FIG. 7.—Appearance of inoculated S-37 cells in the peritoneal fluid after 5 days. Aceto-orcein, ×800.

FIG. 8.—Appearance of inoculated S-37 cells in the peritoneal fluid after 8 days. Aceto-orcein, ×800.

1　2　3
4　5　6
7
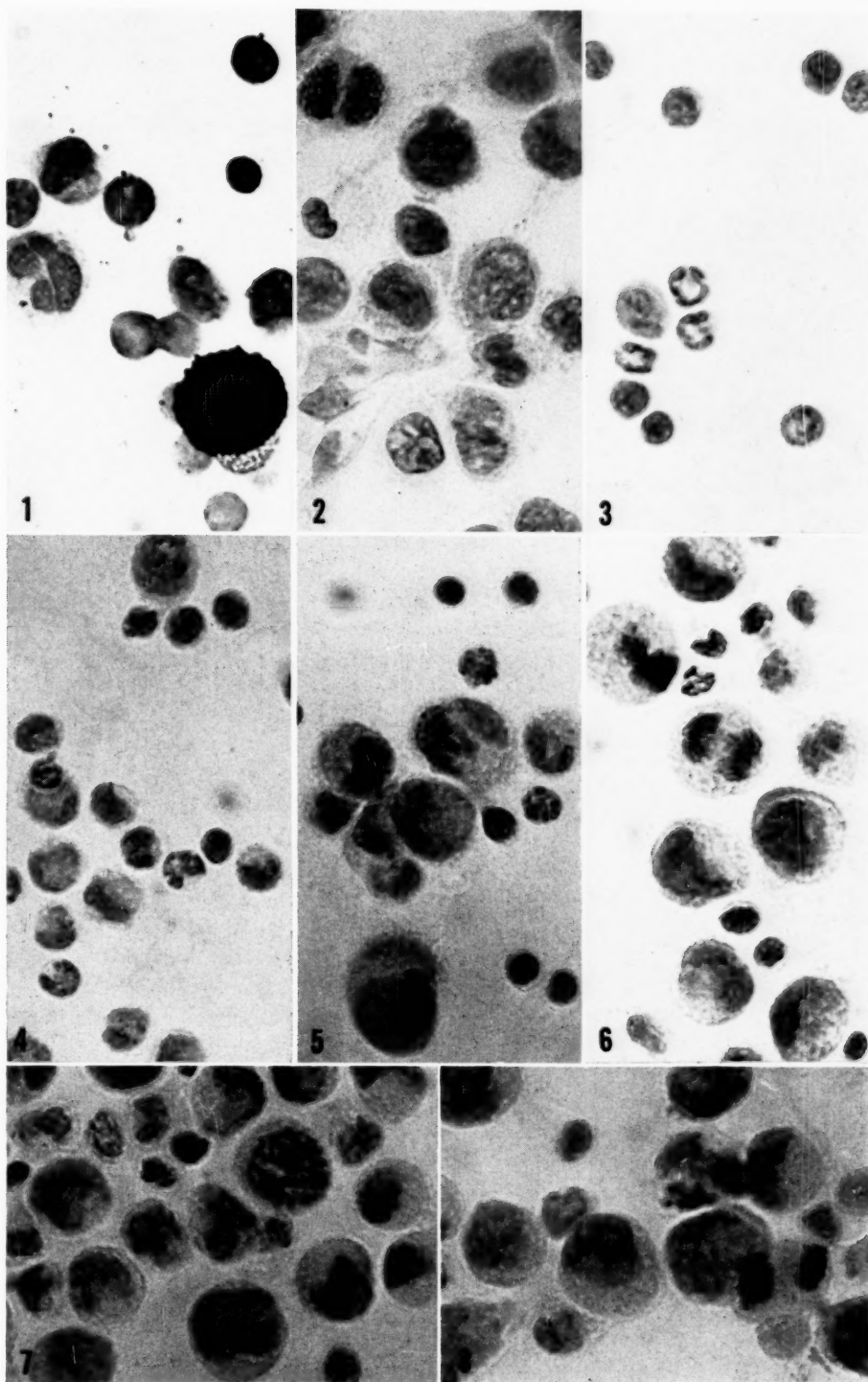
FIGURES 9–14.—Appearance of malignant lymphoma cells from subcutaneous implants in various stages of their growth cycle in the peritoneal fluid.

FIG. 9.—Peritoneal fluid from a dba mouse before inoculation. Wright, ×800.

FIG. 10.—Malignant lymphoma mash (from 14-day-old subcutaneous implant) used for inoculation. Wright, ×800.

FIG. 11.—Same. Aceto-orcein, ×800.

FIG. 12.—Malignant lymphoma cells in the peritoneal fluid of a dba mouse 8 days after inoculation of 10,000 cells.

FIG. 13.—Malignant lymphoma cells in the peritoneal fluid of a dba mouse 12 days after inoculation of 10,000 cells.

FIG. 14.—Malignant lymphoma cells in the peritoneal fluid of a dba mouse 14 days after inoculation of 10,000 cells.
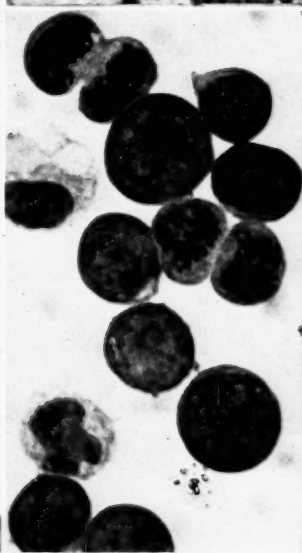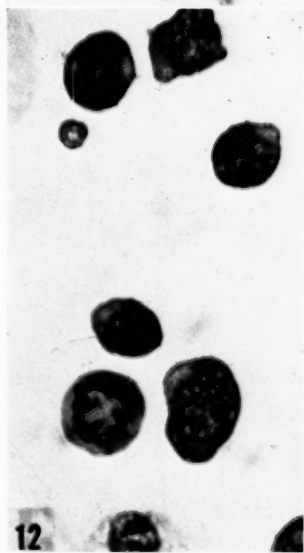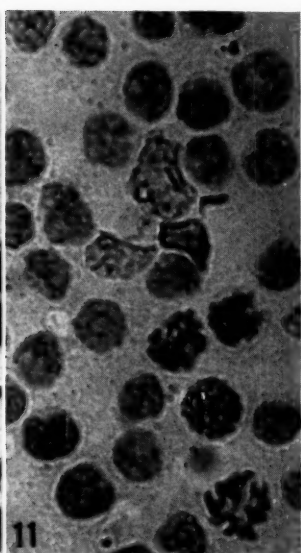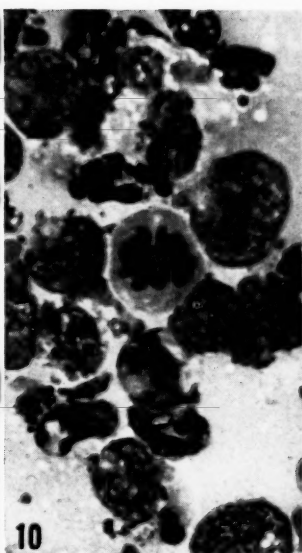
FIGURES 15–20.—Growth cycle of S-37 cells in the peritoneal fluid of a CFW mouse after the 40th intraperitoneal transfer of 10,000 cells. Aceto-orcein, ×800.

FIG. 15.—Appearance of the cells after 6 hours.

FIG. 16.—Appearance of the cells after 24 hours.

FIG. 17.—Appearance of the cells after 48 hours.

FIG. 18.—Appearance of the cells after 72 hours.

FIG. 19.—Appearance of the cells after 6 days.

FIG. 20.—Appearance of the cells after 10 days.

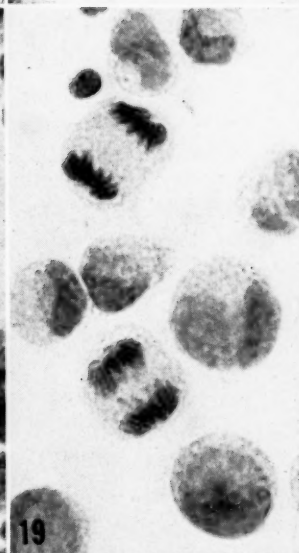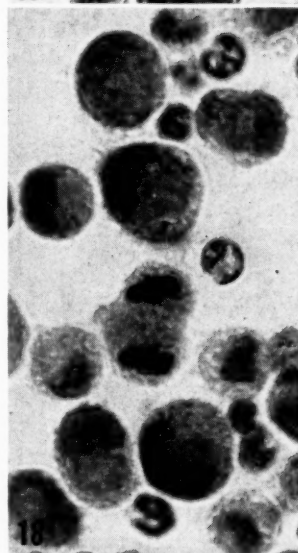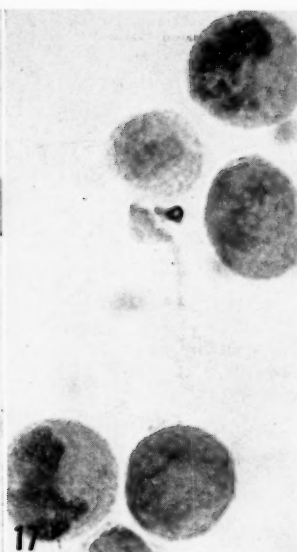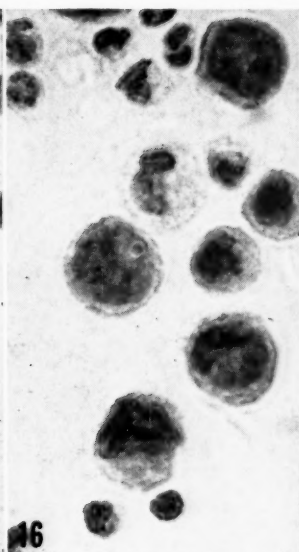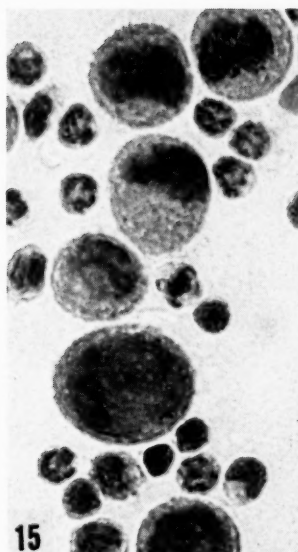FIG. 21.—Lymphoma cells in the peritoneal fluid of a dba mouse 10 days after the 19th serial transfer of 1,000 cells. Aceto-orcein, ×800.

FIG. 22.—Lymphoma cells in the peritoneal fluid of a dba mouse 14 days after the 19th transfer of 100 cells. Aceto-orcein, ×800.
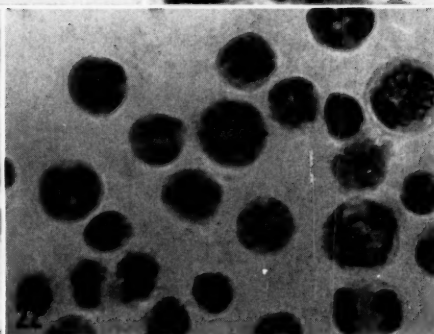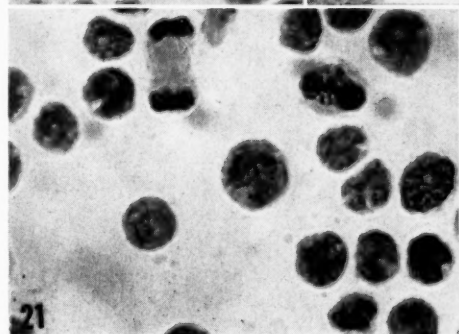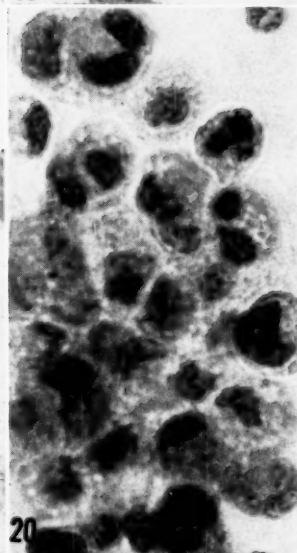
The Changes in Cell Morphology and Histochemistry of the Testis Following Irradiation and Their Relation to Other Induced Testicular Changes*

II. Comparison of Effects of Doses of 1,440 r and 5,050 r with 300 r

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The previous report (2) on quantitative random sampling of the frequency of germinal elements in adult male mice post-radiation at the 300-r dose level emphasized the need for further study of the biological effects of radiation at larger doses. Doses of 1,440 r and 5,050 r were chosen for the present study, because it was hoped that the dosage necessary to cause permanent loss of all the germinal elements would be somewhere between these levels.

An extensive review of the literature was included in the first paper of the series (2).

Results obtained in the earlier studies clearly indicated that more information concerning larger doses should be compiled for each germinal element in reference to (a) timing of onset of disappearance, (b) time required to produce maximum effect, (c) duration of maximum effect, (d) time and extent of recovery, and (e) numerical variations in animals studied.

It was also desirable to give a dose which would cause complete sterility, in order to gain information concerning the thesis that germinal epithelium may have an endocrine function, as discussed by Liebow, Warren, and DeCoursey (3).

It was hoped, too, that a dose causing complete sterility might yield data concerning the suggestion of Eschenbrenner and Miller (1) that degeneration of Sertoli cells following radiation is a contributing factor in the decrease of spermatozoa and degeneration of spermatids.

The data presented here are considered to contribute to a better understanding of growth. First, this work deals with the biological effects of radiation on the progressively differentiating cells in normal spermatogenesis; the embryonal-type cell is represented by the spermatogonia and the highly differentiated type cell by the sperm. Second, this study offers a comparison of the reaction of non-germinal tissue which has not been visibly affected by radiation.

METHODS OF PROCEDURE AND OBSERVATIONS

The testes of 78 pure-line, young, adult C57 black mice, weighing 18–25 gm., were subjected to a single exposure of 1,440 r with the following factors: 100 KvP, 15 Ma Hvl, 2.6 mm. Al, 187 r per minute at TSD of 20 cm. For another set of 74 animals, the acute dose was 5,050 r. The method of procedure was to radiate the testes only, with the body of the animal protected by a lead shield. Two mice were killed on each of the days indicated on Charts 1–4 and 5–8 and on days 56 and 84, with the following exceptions: ten mice of the 1,440-r series were killed on days 4, 12, 16, 26, and 42 and ten of the 5,050-r series on days 4, 8, and 14.

The quantitative random sampling method used was described in the first paper of this series (2) and may be reviewed here. Slides representative of the whole of both testes were studied at a magnification of 430 ×, and from each animal 100 carefully selected median cross sections of tubules were chosen. Each cross section was observed for the presence of each of the four germinal elements. As indicated above, two or ten pairs of testes were studied for each interval recorded, and the numbers given for germinal elements in Charts 1–4 and 5–8 thus represent the average of the counts of two or ten animals.

CHART 1

SPERMATOGONIA
1440r

POSITIVE FIELDS

-5 -4 -3 -2 -1 0 1 2 3 4 5   10   15   20   25   30   35   40
DAYS AFTER RADIATION

CHART 5

SPERMATOGONIA
5050r

POSITIVE FIELDS

-5 -4 -3 -2 -1 0 1 2 3 4 5   10   15   20   25   30   35   40
DAYS AFTER RADIATION

CHART 2

SPERMATOCYTES
1440r

POSITIVE FIELDS

-5 -4 -3 -2 -1 0 1 2 3 4 5   10   15   20   25   30   35   40
DAYS AFTER RADIATION

CHART 6

SPERMATOCYTES
5050r

POSITIVE FIELDS

-5 -4 -3 -2 -1 0 1 2 3 4 5   10   15   20   25   30   35   40
DAYS AFTER RADIATION

CHART 3

SPERMATIDS
1440r

POSITIVE FIELDS

-5 -4 -3 -2 -1 0 1 2 3 4 5   10   15   20   25   30   35   40
DAYS AFTER RADIATION

CHART 7

SPERMATIDS
5050r

POSITIVE FIELDS

-5 -4 -3 -2 -1 0 1 2 3 4 5   10   15   20   25   30   35   40
DAYS AFTER RADIATION

CHART 4

SPERM
1440r

POSITIVE FIELDS

-5 -4 -3 -2 -1 0 1 2 3 4 5   10   15   20   25   30   35   40
DAYS AFTER RADIATION

CHART 8

SPERM
5050r

POSITIVE FIELDS

-5 -4 -3 -2 -1 0 1 2 3 4 5   10   15   20   25   30   35   40
DAYS AFTER RADIATION

Chart 1 illustrates graphically the numerical frequency of spermatogonia following a single dose of 1,440 r. It will be noted that the number of cross sections of tubules showing gonia decreased during the first 24 hours. This decrease is marked for about 5 days, and in 10 days the low point is reached. Recovery is apparent by 28 days, but for the extent of the experiment (42 days) some cross sections of tubules show no evidence of recovery. On the average, only 24 cross sections of 100 per animal show spermatogonia, while the normal untreated testes will show an average of 80. The black area in this figure and those following represent the extent of numerical variation. For example, if ten animals were used for study at a given interval, there would be at least one animal showing the maximum numerical frequency. The numerical frequency of each of the ten animals is averaged, and this average is represented by the white line in the graphs. In our observations, there was little frequency variation for the spermatogonia following a single dose of 1,440 r. This numerical evidence of lack of variation continued until about 35 days post-radiation.

Charts 2, 3, and 4 show the numerical frequency of spermatocytes, spermatids, and sperm, respectively, following a single dose of 1,440 r. They illustrate not only the numerical frequency of any germinal element at any given interval and the extent of variation among animals which have had the same treatment but indicate (*a*) onset of disappearance of any element, (*b*) time required to produce the maximum effect, (*c*) time when recovery starts, (*d*) extent of recovery, and (*e*) the variability in counts from day to day, as indicated by the peaks and depressions.

Chart 2 shows that the number of spermatocytes is not reduced until about 6 days post-radiation. This is in contrast to the spermatogonia which started to disappear in 24 hours. The disappearance period is about 5 da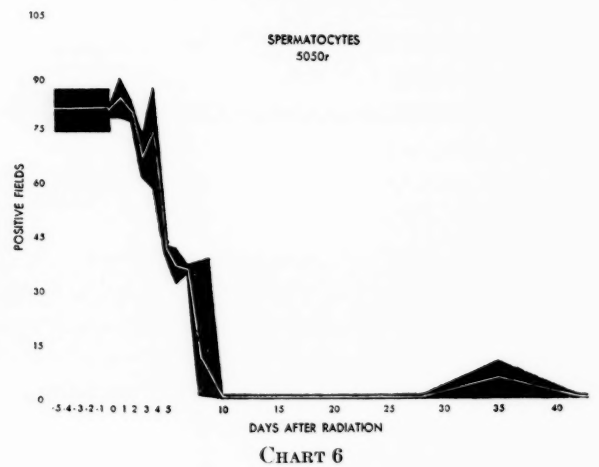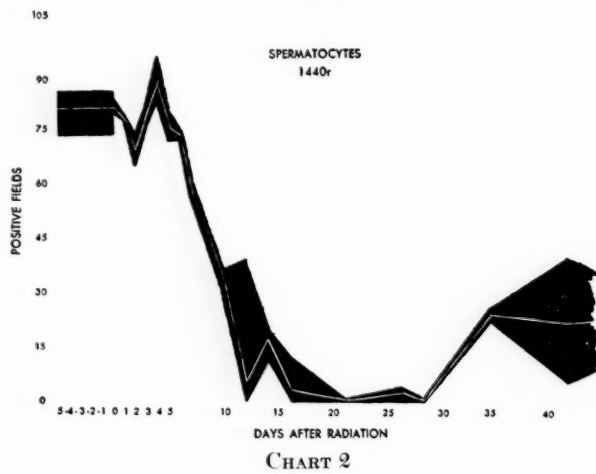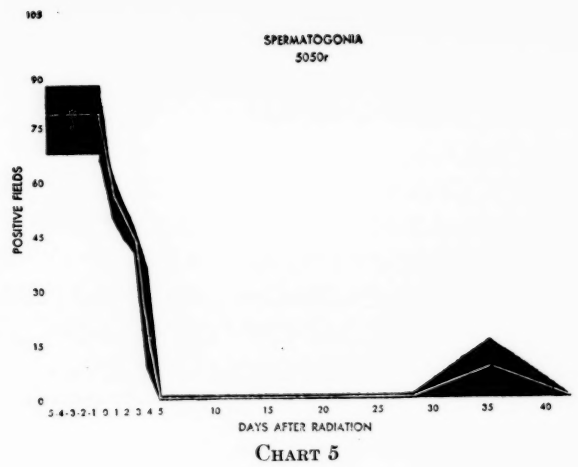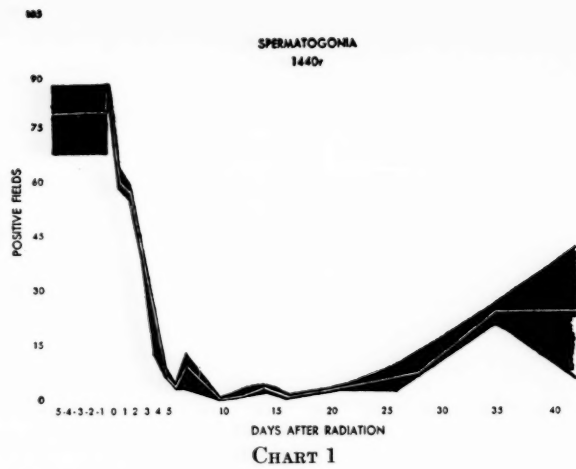ys, and this is the same as for spermatogonia. The low point for spermatocytes, therefore, is around 12 days. This status continues until after the 28th day. Recovery is never complete during the period of the experiment.

Chart 3 shows that spermatids do not begin to decrease consistently until about the 14th day, and they reach their low point by the 21st. Attempted recovery is apparent after the 12th day but is incomplete in the time period. In the spermatids, marked peaks and depressions are noted in the early periods post-radiation.

Chart 4 shows that disappearance of sperm is marked after 21 days and lasts for about 6 days, after which a recovery trend is clear. Peaks and depressions and wide variation in frequency of counts among animals are to be noted. These observations are summarized in Table 1.

It can be seen that the onset of disappearance is progressively later for spermatogonia, spermatocytes, spermatids, and sperm as a result of direct x-radiation with a dosage of 1,440 r. This also occurred after a dose of 300 r but the changes are more prolonged. This quantitative random sampling also indicates that the period of decreasing frequency of each of the germinal elements is approximately of the same duration. The time it takes to produce the maximum effect is roughly the same for the undifferentiated embryonal spermatogonia, spermatocytes, spermatids, and differentiated sperm. This is indicated in the figures in the slope of the curve. At this dose of 1,440 r, there is no evidence of recovery until the 28th day. On the 28th day, however, all the germinal elements begin to recover, regardless of time when radiation effects were first noted.

This study was extended to 84 days to determine whether or not the degree of recovery would approach normal. Results of counts in these later periods (which do not appear in the figures) indicate that there is a gradual increase in the presence of all the germinal elements, but there is no evidence of complete recovery. At 84 days post-

CHART 1.—Numerical frequency of spermatogonia, as determined by quantitative random sampling plotted against time following direct radiation of testis, 1,440 r. The black area in these charts represents the extent of numerical variation. The numerical frequency at each interval of each of the two or ten animals is averaged, and this average is represented by the white line in the graphs.

CHART 2.—Numerical frequency of spermatocytes, as determined by quantitative random sampling plotted against time following direct radiation of testis, 1,440 r.

CHART 3.—Numerical frequency of spermatids, as determined by quantitative random sampling plotted against time following direct radiation of testis, 1,440 r.

CHART 4.—Numerical frequency of sperm, as determined by quantitative random sampling plotted against time following direct radiation of testis, 1,440 r.

CHART 5.—Numerical frequency of spermatogonia, as determined by quantitative random sampling plotted against time following direct radiation of testis, 5,050 r.

CHART 6.—Numerical frequency of spermatocytes, as determined by quantitative random sampling plotted against time following direct radiation of testis, 5,050 r.

CHART 7.—Numerical frequency of spermatids, as determined by quantitative random sampling plotted against time following direct radiation of testis 5,050 r.

CHART 8.—Numerical frequency of sperm, as determined by quantitative random sampling plotted against time following direct radiation of testis, 5,050 r.

radiation, the average frequency of appearance for spermatogonia was 52, as compared to 80 for the normal; 47 for spermatocytes, as compared to 82; 49 for spermatids, as compared to 88; 31 as compared to 85 for the sperm.

The quantitative random sampling of the germinal elements following direct x-radiation at 1,440 r as compared to 300 r showed some similarities. The rate of disappearance as indicated by the slopes of the curves is roughly the same; all the germinal elements are not destroyed, and the decrease of spermatogonia, spermatocytes, spermatids, and sperm occurs in succession after radiation. By the same procedure, our results show some dissimilarities. For example, 1,440 r cause greater permanent injury to the tubules than was observed after 300 r. There are less conspicuous peaks and depressions in the spermatogonia and

there were no spermatogonia, and no permanent recovery was indicated after that time.

The slope of the curve indicating decrease in frequency for the spermatocytes is approximately the same as that reported for 300 r and 1,440 r. After 12 days, no spermatocytes were found, and there was no evidence of permanent recovery.

The spermatids (Chart 7) retain their normal frequency for approximately 2 days and then progressively decrease until complete disappearance at the 14th day.

The sperm did not begin to decrease until the 4th day and were lacking completely by the 21st day (Chart 8).

It may be noted that there is a period post-radiation, 5,050 r, when no germinal element may be found in the testes. This is the equivalent of sterilization. Spermatogonia, spermatocytes, sper-

TABLE 1

CHANGES IN THE GERMINAL ELEMENTS OF MOUSE TESTES FOLLOWING X-RADIATION WITH 1,440 r

	Spermatogonia	Spermatocytes	Spermatids	Sperm
Onset of disappearance	24 hours	6th day	14th day	21st day
Duration of decrease in frequency	5–6 days	5–6 days	5–6 days	5–6 days
Low point	6–28 days	12–28 days	21–28 days	28–29 days
Recovery	Incomplete	Incomplete	Incomplete	Incomplete
Period of greatest numerical variation	Little variation	10–15 days	10–20 days	12–25 days

possibly in the spermatocytes. This is illustrated in the charts.

About 28 days post-radiation, at 1,440 r, all the germinal elements show an almost simultaneous increase in frequency of appearance. This consistent feature was not apparent in the data obtained from the study of the material post-radiation, 300 r. In that experiment, the period of recovery was progressively later for each of the germinal elements from spermatogonia to sperm.

The charts, previously published for 300 r, showed that the greatest numerical variation for any germinal element fell relatively in the same periods, post-radiation. When the dose was increased to 1,440 r, the period of greatest variation became progressively later for the spermatocytes, spermatids, and sperm. The period of greatest numerical variation for each of the germinal elements is indicated in the preceding table.

In another set of experiments, 74 animals were given a single dose of 5,050 r. This was chosen because it was noted that the single dose of 1,440 r had not caused complete sterilization. Charts 5–8 show the results of the quantitative sampling of germinal cells at intervals following a single dose of this magnitude.

The spermatogonia were the first to show decrease in frequency following radiation. At 5 days

matids, and sperm become completely depleted from the tubules at progressively later intervals at 5–10–14–21 days, respectively.

Once the disappearance of any germinal element begins as a result of radiation, the maximum expression of this phenomenon takes approximately 5–6 days. This 5–6-day interval is consistent for each of the germinal elements. In other words, it takes a spermatogonium and a sperm approximately the same time to reach their low point of incidence from the time that decrease in frequency begins. This slope of the curve is approximately the same for each of the germinal elements and is unchanged when dose is varied. For example, after an interval of 5–6 days post-radiation, 5,050 r, no spermatogonium can be found in the tubules. Five days after the first evidence or marked decline in occurrence of spermatogonia, following a dosage of 1,440 r, one cross section out of 100 revealed gonia; likewise, for this interval after 300 r, 10 out of every 100 cross sections revealed gonia. This represents the maximum effect after each dose. In summary, the rate of disappearance may be approximately the same, but the extent of the damage is relative to dosage.

An examination of the table and charts for 1,440 r, 5,050 r, and reference to the report on 300 r show some similarities and some differences.

A single dose of 5,050 r is similar to 300 r and 1,440 r in that the slope of the curves representing the period of partial or complete disappearance of each germinal element is approximately the same. After each of the three dosages the spermatogonia are the first to show radiation effect, followed progressively by the spermatocytes, spermatids, and sperm. Some diversity in the peaks and depressions in the graphs and numerical variation are also apparent. The main observable difference between 5,050 r, and 1,440 r and 300 r is the degree of destruction of the germinal elements.

Some of the mice died before the date when they would have been sacrificed, but the death rate at no time seemed excessive for the lower dosages.

Examination of the slides after 21 days postradiation, 5,050 r, or that period which could be considered agerminal, revealed no morphological alterations of the Sertoli cells nor any marked reduction in number. The interstitial cells appear normal. No mitoses have been noted during the period of 84 days post-radiation. Blood vessels and connective tissue do not show any marked pathological change during this period.

DISCUSSION

The doses of 1,440 r and 5,050 r were selected in the hope that these would encompass the dose which would destroy all the germinal elements. It was also of interest to compare different doses, with all other conditions held constant. We have found that for young adult (18–25 gm.) C57 black mice, direct exposure to 1,440 r does not cause sterilization, but a dose of 5,050 r will destroy all the germ cells.

It has been shown that 300 r destroys roughly 90 per cent of the germinal elements, while 1,440 r destroys 97–98 per cent, and a single dose of 5,050 r goes beyond the necessary level for complete lysis of the germinal cells, excluding the Sertoli cells. From this work it might be estimated that a dose in the range of 2,000 r would be the minimal dose level for destruction of germinal elements. Data are being accumulated in an attempt to establish the critical dosage.

An intimation of comparative sensitivity, based on the time required for each of the germinal elements to decrease in frequency of appearance for a single dose of 5,050 r, is shown in Charts 5–8. As indicated, all the gonia disappear in 5 days. The spermatocytes disappear in 10 days, the spermatids in 14 days, and the sperm in 21 days. This progressive disappearance of each of the germinal elements represents the same sequence that occurs in spermatogenesis and thus suggests a correlation between sensitivity and cell specialization in development.

The 1,440-r results demonstrate clearly that some parts of the testes are permanently injured. The quantitative random sampling reveals that after an interval of about 25 days only 2 or 3 cross sections of tubules of 100 in a given testis show the germinal elements. This is defined as the maximum effect, and the residuum of viable cells are the potential regenerative cells. The average frequency for each of the germinal elements at later intervals (42–52 days) showed that 40 cross sections of 100 showed germinal elements, in contrast to 80 of 100 cross sections in a normal testis. This suggests that 1,440 r have destroyed approximately one half of the regenerative capacity of the testis. It does not indicate whether the remaining half-capacity of the testis can produce normal sperm.

Because cross sections of 2–3 tubules reveal gonia, it is possible that these gonia had not been affected by the radiation. If this were so, breeding experiments could produce successful matings. A successful mating is defined as the production of normal phenotypes by a nonradiated female mated to a radiated male. One successful mating resulted from several breeding experiments.

The direct exposure of the testes to 5,050 r should give an opportunity for determining whether the absence of germ cells is a cause for endocrine imbalance. Unfortunately, the survival factor for these radiated animals was low; by 12 weeks after radiation all the animals had died. This failure to survive was not anticipated, and its cause is now being investigated.

In 84 days, after a dose of 5,050 r, there was no reduction in the number of Sertoli cells or obvious pathological change in them. Neither was there any mitotic activity in the interstitial cells. It is possible that at intervals later than those included in this study, interstitial cells might multiply and produce tumors, but it has not been observed. There was no regeneration noted of spermatogonia from other cells not designated as such—as, for example, from connective tissue in the capsule or from Sertoli cells.

SUMMARY

Evidence is presented to show that a dose of 1,440 r does not destroy all the germinal elements at any time; there is a sequence for radiation reaction from gonium to sperm, and there is an eventual reduction of production.

In comparison to 300 r, it is indicated that a single dose of 1,440 r further reduces the frequency of the appearance of the germinal cells and delays

the time of recovery for spermatogonia and spermatocytes. There is also less evidence of numerical variation in the spermatogonia and spermatocytes and less marked peaks and depressions in the figures.

An acute dose of 5,050 r destroys all the germinal elements, but all these elements do not disappear at the same time. The gonia disappear first, followed by the spermatocytes, spermatids, and sperm.

Once each of the types of germinal elements starts to disappear, the rate is approximately the same, regardless of the dose.

The degree of injury is relative to the magnitude of the dose.

No marked pathological change was noted in cells or tissues other than in the true germinal cells.

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Chemical Alterations Induced in Mouse Liver Following a Single Feeding of Carbon Tetrachloride*

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The chemical processes involved in cellular damage and regeneration are of importance in neoplasia. Agents such as carbon tetrachloride produce a sequence of liver damage, regeneration, and hepatoma formation. A single feeding of carbon tetrachloride produces extensive liver necrosis in mice, followed by a rapid proliferation of the remaining uninjured cells, until a normal complement of functioning tissue is restored (14). Because of the large number of liver cells rendered necrotic by carbon tetrachloride, an extensive synthesis of cellular constituents must necessarily be required prior to a complete restoration of this tissue. Relatively little is known regarding the nature of synthetic processes resulting in the growth of cells. An extensive series of investigations concerning the relationships between nucleic acids and the synthesis of protein has been reviewed by Caspersson (6, 7).

A chemical characterization of the necrosis and subsequent regenerative growth induced in mouse liver by carbon tetrachloride is complicated by a considerable overlapping between the two processes. Because of this complication, rigid histopathological control is necessary for the proper interpretation of the chemical findings. In view of the dynamic nature of tissue degeneration and regeneration, a chemical characterization of these phenomena requires a number of investigations at various intervals throughout the extent of these processes.

A series of experiments has been undertaken to investigate cellular necrosis and regenerative growth induced in mouse liver by feeding of carbon tetrachloride (18, 30). Some chemical alterations found to be associated with these processes will be described in this paper. The investigations included analyses for water, lipids, and nucleic acids, as well as a general study of the distribution of nitrogen and phosphorus. The investigations to be reported were conducted in correlation with thorough histological and histochemical studies (29). Experiments were devised so that, following a single feeding of carbon tetrachloride, mice were sacrificed at various intervals until all degenerative and regenerative processes appeared to be complete.

EXPERIMENTAL

All experiments were performed on male strain A mice, 3 months of age. Each experimental animal received a single feeding of 0.1 ml. of 40 per cent carbon tetrachloride in olive oil by stomach tube (14). Groups of mice were sacrificed at intervals of 1, 2, 3, 4, 5, 6, 12, and 18 days following the carbon tetrachloride feeding. All mice utilized in these experiments weighed $24 \pm .5$ gm. at the start of each experiment and were starved 18 hours prior to sacrifice.

After the specified time intervals following carbon tetrachloride feeding, the animals were anesthetized with ether, and their livers were removed. Prior to the removal, the portal vein was clipped while the heart was still beating, which resulted in a visible blanching of the liver. The excised, bled livers were blotted with gauze and immediately weighed. Representative samples were removed for histological fixation. The remaining tissue was re-weighed and transferred to an all-glass homogenizer (Scientific Glass Co.) and ground in a volume of distilled water equivalent to 9 ml. per gram of tissue. Individual as well as pooled liver samples were prepared in this manner. All tissues were maintained as close to $0°$ C. as possible, following their removal. Immediately following the preparation of the liver homogenates, measured aliquots were removed for a number of chemical analyses.

General Histological Findings

Quantitative cytological measurements (8) disclosed a necrosis of nearly 40 per cent of the total liver within 2 days subsequent to the carbon tetrachloride feeding. The bulk of the necrotic tissue

was present until the fourth day in association with inflammatory changes, following which time it was rapidly removed; little necrosis was evident by the sixth day. Regenerative changes meanwhile were evident from the second day. A maximum number of mitotic cells was found on the second and third days; fewer mitoses were present after the fourth day. Most mitotic activity was therefore essentially complete prior to the removal of the bulk of necrotic tissue. From the histological study, therefore, considerable overlapping between the necrosis and regenerative processes was evident on the second, third, and fourth days after the carbon tetrachloride feeding. Past the fourth day most changes could be attributed to the regenerative process.

ured aliquots of the liver homogenates by micro procedures similar to those proposed by Bloor (1). The method involved a thorough extraction of the homogenized tissues with hot alcohol-ether (3:1), followed by a further isolation of the extracted lipids by re-extractions with petroleum ether. The petroleum ether re-extracts were further purified by repeated washings with water to remove adsorbed nonlipid contaminants (16). Following the evaporation of petroleum ether, the extracted lipids were dried to constant weight *in vacuo* over phosphorus pentoxide.

The results of these investigations (Table 1) show a marked increase in liver weight following carbon tetrachloride feeding. Increases in water content and, in general, corresponding decreases in

TABLE 1

ALTERATIONS IN WEIGHT, WATER, AND LIPID CONTENT OF MOUSE LIVER FOLLOWING CARBON TETRACHLORIDE ADMINISTRATION

No. days following carbon tetrachloride feeding	Total no. of mice	Av. body weight (gm.)	Av. liver weight (gm.)	Av. liver weight to body weight ratio	Av. moisture content		Av. lipid content		Av. liver wt. minus water and lipid (gm.)
					Per liver (gm.)	Per cent total liver	Per liver (gm.)	Per cent total liver	
0 (Control)	21	21.9	1.09	4.98	.662	60.7	.177	16.2	.25
1	11	21.3	1.25	5.87	.827	66.2	.186	14.9	.24
2	11	19.8	1.46	7.37	.984	67.5	.210	14.4	.26
3	18	20.6	1.61	7.82	1.06	65.7	.219	13.6	.33
4	17	19.7	1.35	6.85	.942	69.7	.107	7.94	.30
5	15	20.0	1.25	6.25	.866	69.3	.077	6.22	.31
6	17	20.8	1.19	5.72	.813	68.4	.084	7.03	.29
12	11	22.0	1.14	5.18	.761	66.7	.105	9.17	.27
18	8	22.7	1.14	5.02	.716	62.7	.151	13.2	.27

CHEMICAL ANALYSES AND RESULTS

Alterations in size, water, and lipids.—Gross examination of the livers removed from the experimental animals disclosed a marked increase in liver size following the administration of carbon tetrachloride. In view of the extreme alterations in water and lipid content associated with many forms of liver pathology, an infiltration of one or both of these constituents was suspected. Experiments were performed to determine the extent of alteration in weight, water, and lipid content of mouse liver following carbon tetrachloride feeding.

Ratios of liver to total body weight were determined for each experimental animal at the time of sacrifice. From these values it was possible to determine alterations in liver size. The water content of the experimental livers was estimated by drying measured aliquots of the homogenized tissues at 105° C. for at least 2 hours. Longer drying times at this temperature, as well as drying at 65° C. *in vacuo* for 24 hours, were found to show essentially the same values.

Lipids were extracted and isolated from meas-

liver lipid were found to accompany the increases in liver weights. After computing the liver weights, therefore, on a lipid-free dry basis, increases in the weight of this organ were still evident. By computing the average liver weights on this basis, a maximum increase of 32 per cent was found over the control values. This maximum increase in average lipid-free dry liver weight was followed by a gradual reduction to nearly normal weights by the eighteenth day. The increases in liver weights up to the fourth day could be attributed for the most part to a restoration of liver tissue at a rate in excess of the removal of necrotic cells. The increases subsequent to the fourth day, by which time the bulk of necrotic tissue had been removed, were considered a temporary overgrowth of this organ.

Further analyses were performed on the isolated lipid fractions (Table 2) to determine the nature of the marked reduction in these constituents following carbon tetrachloride administration. The dried and weighed lipid fractions were dissolved and made to known volumes in chloroform. Measured aliquots were removed for total cholesterol deter-

minations by an application of the Liebermann-Burchard reaction (2). Other aliquots were removed for phosphorus analyses (15), from which values phospholipid was computed by assuming an average phospholipid of molecular weight 744 (17). The amount of total weighed lipid minus the experimentally determined amounts of cholesterol and phospholipid was calculated to be neutral fat.

Little alteration was found in the cholesterol content of the experimental livers. Analysis for phospholipid indicated an initial decrease followed by some accumulation of these constituents subsequent to carbon tetrachloride feeding. The over-all decreases observed in total liver lipid following carbon tetrachloride administration were attributed principally, therefore, to a reduction in neutral fat. Histological studies disclosed much less lipid in the recently formed liver cells following

TABLE 2

ALTERATIONS IN MOUSE LIVER LIPIDS ASSOCIATED WITH CARBON TETRACHLORIDE ADMINISTRATION

No. DAYS FOLLOWING CCl₄ ADMINISTRATION	No. SEP. EXP.	TOTAL NUMBER OF MICE	LIVER LIPID FRACTIONS		
			Cholesterol	Phospholipid (mg. per liver)	Neutral fat
0 (Control)	7	21	9.15	36.4	131
1	4	11	9.25	30.8	146
2	4	11	12.7	32.9	164
3	5	18	12.6	40.9	166
4	5	17	9.31	38.8	58.9
5	2	15	9.13	40.6	27.3
6	5	17	6.78	40.6	36.6
12	4	11	8.67	40.7	55.6
18	1	8	10.6	39.4	101

carbon tetrachloride feeding (29). Whether the over-all decreases in the liver neutral fat following carbon tetrachloride administration can be attributed entirely to the regenerative process or reflect, in part, a temporary inability of the liver to store this constituent remains to be determined.

Nitrogen and phosphorus distribution studies.— The distribution of nitrogen and phosphorus within liver tissue following carbon tetrachloride feeding was investigated. The study included a separation of the tissues into four chemical fractions (25): cold acid-soluble, hot alcohol-ether-soluble, nucleic acid, and residual protein.

Chemical fractionations of the liver tissues were conducted on 5.0-ml. aliquots of the prepared 1:10 liver homogenates. The measured aliquots were transferred to 12-ml. heavy-walled centrifuge tubes and extracted 3 times with cold 5 per cent trichloroacetic acid. The three extracts containing cold acid-soluble constituents were combined and diluted to volume in 25-ml. volumetric flasks with water. The resulting tissue residues were extracted twice with 4-ml. portions of cold 95 per cent ethyl

alcohol, followed by four extractions with 4-ml. portions of hot alcohol-ether (3:1). The cold alcohol and hot alcohol-ether extracts were combined and diluted to volume in 25-ml. volumetric flasks with alcohol. The resulting precipitates were next heated with 8-ml. portions of 5 per cent trichloroacetic acid at 90° C. for 20 minutes to extract the nucleic acids. Following the heating, the tubes were centrifuged and the precipitates washed twice with 5 per cent trichloroacetic acid. The original extracts and washes were combined and diluted with 5 per cent trichloroacetic acid to 25 ml. in volumetric flasks. The remaining protein residues were dissolved by heating with 1-ml. portions of 1 N sodium hydroxide and diluted with water to 25 ml. in volumetric flasks.

Following the fractionation, measured aliquots were removed from each isolated fraction for nitrogen and phosphorus analyses. Nitrogen was determined by the micro-Kjeldahl procedure of Pregl (22) and phosphorus by the method of Fiske and Subbarow (15).

In view of the marked alterations observed in size and lipid content of mouse liver subsequent to carbon tetrachloride feeding, all results were computed on the basis of total liver (Table 3). Thus, absolute as well as relative alterations in the distribution of nitrogen and phosphorus could be followed. In addition to the analyses on each of the separated fractions, nitrogen and phosphorus were determined for purposes of comparison on the whole unfractionated livers.

Marked accumulations in total liver nitrogen and especially in total liver phosphorus were observed following carbon tetrachloride feeding. By the fourth day a maximum increase of 29 and 55 per cent over control values was found for liver nitrogen and phosphorus, respectively. These increases reflected a number of interesting alterations in their chemical distribution. The accumulation of total liver nitrogen could be attributed to general increases in each of the separated chemical fractions. However, the bulk of accumulated nitrogen was present in the protein fractions and was found to reach a maximum of 25 per cent above normal values by the sixth day. The increases in total liver phosphorus reflect a marked accumulation in cold acid-soluble phosphorus as well as in nucleic acid phosphorus. Further analyses of the cold acid-soluble fractions disclosed the accumulation of phosphorus to be attributable to proportionate increases in both inorganic as well as organically bound phosphate. Some increase in phosphorus and somewhat greater increases in nitrogen were found in the hot alcohol–ether-soluble fractions. Although the greater part of the nitrogen

present in these fractions was nonlipid, essentially all the phosphorus could be attributed to phospholipid.

The accumulation of liver nitrogen following carbon tetrachloride administration parallels in general the increases observed in lipid-free dry liver weights (Table 1). As previously mentioned, the increases in liver weights were attributed to a vigorous regeneration resulting initially in a restoration of tissue at a rate exceeding the removal of

on the isolated hot trichloroacetic acid fractions described in the previous study.

Pentose was estimated in the isolated fractions by the orcinol reaction with suitable corrections (4). Desoxypentose was determined by the method of Dische (12). The respective quantities of nucleic acid were calculated from these determinations on the basis of previous analyses on highly purified preparations of both nucleic acid types isolated from normal mouse liver (32). As an additional

TABLE 3

DISTRIBUTION OF NITROGEN AND PHOSPHORUS IN MOUSE LIVER FOLLOWING CARBON TETRACHLORIDE FEEDING

DAYS AFTER SINGLE FEEDING OF CCl₄	No. SEP. EXP.	TOTAL NO. MICE	Av. LIVER WT. (gm.)	TOTAL LIVER N	P	ACID SOLUBLE N	P	HOT ALCOHOL-ETHER SOLUBLE N	P	NUCLEIC ACID FRACTION N	P	RESIDUAL PROTEIN FRACTION N	P
				(mg. per liver)		(mg. per liver)		(mg. per liver)		(mg. per liver)		(mg. per liver)	
0 Cont.	7	21	1.09	36.9	3.83	2.40	.968	3.97	1.47	2.92	1.25	27.3	.19
1	4	11	1.25	36.7	3.45	1.97	.898	3.34	1.23	3.20	1.10	27.1	.16
2	4	11	1.46	39.2	3.78	2.84	1.11	3.90	1.32	3.17	1.16	28.7	.14
3	4	11	1.50	42.6	4.94	3.03	1.55	5.20	1.54	3.61	1.59	30.0	.21
4	4	11	1.43	47.7	5.94	3.41	2.11	4.82	1.64	4.30	1.86	33.7	.26
5	2	15	1.24	44.4	5.05	3.13	1.35	4.55	1.62	3.98	1.68	32.2	.31
6	4	11	1.20	45.6	5.07	3.00	1.41	4.15	1.64	4.03	1.71	34.1	.27
12	4	11	1.14	42.2	4.67	2.52	1.25	4.14	1.63	3.60	1.50	31.2	.27
18	1	8	1.14	39.5	4.37	2.32	1.11	4.93	1.58	3.01	1.35	28.6	.27

TABLE 4

ALTERATIONS IN NUCLEIC ACIDS IN RELATION TO PROTEIN NITROGEN CONTENT OF MOUSE LIVER FOLLOWING CARBON TETRACHLORIDE FEEDING

DAYS FOLLOWING CCl₄ FEEDING	No. SEP. EXP.	TOTAL NO. MICE	PENTOSE-NUCLEIC ACID (PNA)	DESOXY-PENTOSE-NUCLEIC ACID (DNA)	PROTEIN NITROGEN	TOTAL NUCLEIC ACID By estimation of specific carbo-hydrates	Deter-mined by U.V. absorption	TOTAL NUCLEIC ACID PHOSPHORUS Found	Calculated (PNA-P plus DNA-P)
						mg. per total liver (Av.)			
0 (Control)	7	21	9.12	3.60	27.3	12.7	13.0	1.25	1.24
1	4	11	8.23	3.21	27.1	11.4	10.6	1.10	1.12
2	4	11	8.83	3.10	28.7	11.9	11.3	1.16	1.16
3	4	11	12.2	4.67	30.0	16.9	16.9	1.65	1.59
4	5	17	13.9	4.91	30.2	18.8	19.4	1.77	1.83
5	2	15	13.2	4.60	32.2	17.8	18.4	1.68	1.73
6	5	17	12.6	4.85	32.6	17.5	18.2	1.69	1.71
12	4	11	11.2	4.84	31.2	16.0	16.1	1.50	1.57
18	1	8	10.3	4.28	28.6	14.6	14.6	1.35	1.43

necrotic cells and eventually to a temporary over-growth of this organ. The marked increases in cold acid-soluble and nucleic acid phosphorus, found in association with the regrowth of liver tissue, suggest their importance in tissue restoration.

Nucleic acid studies.—Numerous experiments have been described in the literature concerning nucleic acids and regenerative liver growth in rats following partial hepatectomy (5, 11, 13, 20, 27). In view of the potential significance attached to these compounds with respect to growth mechanisms (6, 7), further analyses were performed for pentose and desoxypentosenucleic acids (Table 4)

check, total nucleic acid was estimated by ultra-violet adsorption technics (31). Calculated as well as experimentally determined values for total nucleic acid phosphorus have been presented for comparison. The calculations were derived from the values for pentose and desoxypentosenucleic acid, assuming phosphorus compositions previously determined on purified preparations of both nucleic acid types (32). Although some discrepancies were observed among the various analyses, the extent to which these differences might be attributed to either experimental error or true alterations in the nucleic acids *per se* cannot be ascertained.

Following initial decreases, marked accumulations in both types of nucleic acids follow carbon tetrachloride feeding. The initial decreases were attributed to the necrotic changes induced by the carbon tetrachloride. Histological studies have disclosed a complete absence of pyroninophilic (24, 29) and Feulgen-positive material (29) in the necrotic cells 24 and 48 hours, respectively, following carbon tetrachloride feeding. Pyronin has been described as a specific stain for pentosenucleic acid (21). The specificity of the Feulgen reagent for desoxypentosenucleic acid appears to be fairly well established (23, 28). In view of the histological finding that nearly 40 per cent of the liver was rendered necrotic, corresponding losses in nucleic acids would be anticipated. Chemical analyses, however, revealed a maximum reduction of only 10 and 14 per cent for pentose and desoxypentosenucleic acids, respectively. These findings suggest a rapid parallel resynthesis of nucleic acids within the remaining uninjured liver cells so that only slight net reductions in these constituents were observed.

Following the initial decreases, a rapid accumulation of both nucleic acids was found. Maximum increases of 52 and 36 per cent over normal values for total liver pentose and desoxypentosenucleic acids, respectively, were found 4 days after the carbon tetrachloride feeding. Desoxypentosenucleic acid was found to reach near maximum values by the third day and to remain essentially constant up to 12 days, following which time some decline was observed. The desoxypentosenucleic acid once formed, therefore, appears to be less susceptible than other constituents to further change. An accumulation in this type of nucleic acid presumably represents an increase in the number of liver cells (19, 33). Contrary to the findings for desoxypentosenucleic acid, the pentosenucleic acid reached maximum values on the fourth day and immediately declined in concentration. The increase in pentose type of nucleic acid was found to be much greater than the increase in desoxypentose type. Increases in pentosenucleic acid have been claimed by Caspersson (6, 7) to be always associated with cellular synthesis of protein. A direct proportionality was concluded by Caspersson to exist between nucleotide concentration and rate of protein synthesis in all bacteria investigated.

Values for protein nitrogen have been included, in Table 4, with the nucleic acid findings, for the purpose of comparing the relative quantities of each present at the various time intervals studied. Pentosenucleic acid was found to show a maximum accumulation on the fourth day and a subsequent decline in concentration as protein nitrogen continued to accumulate. These observations suggest but do not necessarily establish that protein synthesis is proceeding at the expense of pentosenucleic acid. In view of the simultaneous removal of large quantities of necrotic tissue, presumably containing a proportionate quantity of protein and little pentosenucleic acid between the fourth and sixth days, a synthesis of new protein at a rate even in excess of this removal must be concluded. Quantitative cytological measurements by methods proposed by Chalkley (8) on sections prepared from liver samples were utilized in these studies. These measurements indicated a reduction in the necrotic and inflammatory area from approximately 33 per cent of the total liver area on the fourth day to approximately 5 per cent on the sixth day. Experiments have been reported (26) in which ribonucleic acid was found to be expended during the assimilation of nitrogen by yeast. A marked increase in the turnover rate of the phosphorus in this nucleic acid was found to accompany the assimilation process. Little evidence was apparent from the present studies to support the postulated role of pentosenucleic acid as a precursor in the synthesis of desoxypentosenucleic acid (3, 10). Both nucleic acids appeared to reach maximum values at about the same time, with pentosenucleic acid subsequently being rapidly expended with no further synthesis of desoxypentosenucleic acid apparent. However, in view of the complex nature of the experimental conditions, some reservation must be attached to these conclusions.

GENERAL DISCUSSION

Methods for inducing regenerative growth in tissues such as liver involve a preliminary destruction or extirpation of a certain proportion of the total mass of cells. Extirpation methods appear to have been widely applied in the study of this phenomenon. In view of the necessary general injury induced by either surgical removal or chemical destruction of a large quantity of tissue, chemical characterizations of the subsequent regenerative process may be complicated by alterations induced solely by the injury. Of significance in this respect is an observed marked accumulation of lipids in actively regenerating mouse liver following partial extirpation[1]. In contrast, a marked reduction in liver lipid was found to be associated with regenerative growth following carbon tetrachloride poisoning. From the two separate investigations, changes in the amount of lipid should perhaps be excluded as an alteration specific to the regeneration process *per se*, and should rather be attributed to a response to a specific injury.

[1] Observations to be published from this laboratory.

The processes of initial necrosis and subsequent regeneration in mouse liver were accompanied by numerous chemical alterations. Of special interest was the marked accumulation in total liver phosphorus, reflecting sharp parallel increases in acid-soluble and nucleic acid phosphorus. Further analyses of the acid-soluble fractions disclosed proportionate increases in both inorganic and organic phosphates. Analyses for phosphocreatine on some of the samples indicated an approximate three- to fourfold increase by the third day. In view of the recognized energy-yielding reactions associated with many phosphate compounds present in cold acid extracts of tissues, an increase in their concentration would perhaps imply their importance in the synthesis of cellular constituents. The parallel increases in cold acid-soluble and nucleic acid phosphorus might also imply an accumulation of the former as building units for the rapid synthesis of the latter.

Although some accumulation of phospholipid was associated with the regenerative process, the increases were much less than those observed in the other phosphate fractions. This observation might be correlated with a depression in the rate of phospholipid turnover during regenerative liver growth in rats following partial hepatectomy, reported by Chargaff, Olson, and Partington (9). A considerable increase in phosphorus in the residual protein fractions also accompanies the regeneration process, suggesting the presence of an active phosphate fraction. The phosphate present in these fractions has been considered to represent phosphoprotein phosphorus (25).

In view of the dynamic nature of the chemical changes occurring following the carbon tetrachloride feeding, some difficulty is encountered in attempting to demonstrate the true extent of these alterations. Chemical analyses on many individual liver samples indicated some variation in rates of response by the mice to the administered toxic agent. Therefore, attempts to determine chemical changes on a number of mice following a given time interval can only represent a conservative estimate of the true extent of these alterations.

SUMMARY

A general chemical characterization of the processes of liver degeneration and regeneration in mice following a single feeding of carbon tetrachloride has been presented. An increase in water and corresponding decreases in the level of liver neutral fat were found to accompany these processes. Considerable overlapping between the initial necrosis and subsequent regenerative process was evident. This resulted in a net increase in tissue bulk, due, presumably, to a restoration of tissue at a rate in excess of the removal of necrotic cells.

Studies on the distribution of nitrogen and phosphorus following carbon tetrachloride administration were presented and discussed in relation to the degenerative and regenerative processes.

Nucleic acid analyses indicated some loss of both pentose and desoxypentose types to be associated with liver necrosis, and considerable increases in both to be related to the restoration process. A comparison of the quantitative relationships between pentosenucleic acid, desoxypentosenucleic acid, and protein nitrogen levels at various intervals throughout the active phase of liver regeneration was presented and discussed.

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Spontaneous Gastric Neoplasia in Mice of the Br-S Strain Incidence and Genetic Linkage Tests[*]

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INTRODUCTION

Several differing strains of mice have been produced from the NHO stock (7) as a result of sib-inbreeding associated with repeated subcutaneous injections of methylcholanthrene. One of these strains, Br-S, is characterized by a high incidence of gastric adenomas or adenocarcinomas. The evidence for a hereditary basis of the abnormality is extensive (7–10). However, the etiology is not clear; studies of the gastric pathology (2–4, 6, 11) in this strain indicate considerable variability, and the term "lesion" has often been used instead of tumor. Andervont (1) also uses the term "lesion" for the adenomatous condition characteristic of the I strain. The latter is not the same as that of the Br-S strain in two respects: (*a*) position of origin—at the forestomach junction in strain I mice, but near the pylorus in Br-S; and (*b*) inheritance—outcross F_1 hybrids have the lesion in the case of Br-S, but not in the case of I. The two strains have not yet been crossed with one another.

It is the purpose of the present paper to give data on the incidence of gastric neoplasia in an untreated sub-line of the Br-S and a further study of the genetic basis of the condition in an outcross. The term "tumor" will be used, without pathological certainty, but simply in preference to the more ambiguous "lesion." Other kinds of tumors (e.g., lung tumors) and pathological conditions will be omitted from consideration, since they were infrequent and seem to bear little if any relationship to the main problem.

MATERIALS AND METHODS

The untreated sub-line of the Br-S strain was started in 1944 with ♀ 190592 and her two brothers, in the F_{12} generation (see Strong, 1945 [7], Fig. 1, "G and F"). The female and one brother developed tumors of rather large size

(autopsy at $1\frac{1}{2}$ years old or more). The other male died before 1 year old, and no necropsy was obtained. Brother-sister matings have been continued in all generations. Residual genetic segregation may have differentiated slightly differing sublines within this descent, but all the descendants of the above three mice are here grouped together. Selection has not been consciously practiced.

The care of the mice is the same as described earlier (7). Some of the mice were arbitrarily chosen as breeders, while others (a greater number) were segregated as to sex ("nonbreeders"). The breeders were permitted to raise from one to four litters of young, and after that their young were discarded soon after birth. The breeders were allowed to die of "old age" or were killed when obviously declining in health; the nonbreeders were killed more or less randomly at an average age of about 1 year, although about 12 per cent died. Another difference between the breeders and nonbreeders was that only two to four breeders were kept together, while the nonbreeders were kept in groups of five or occasionally more. In either case, as mice died no replacements were made.

In the genetic tests, which will be more carefully explained below, methods were similar to the above, except that no supplementary foods were given. The majority of these animals was killed at the age of about 540 days.

Necropsy was performed on all the mice except a few which had decomposed and were excluded from the statistics. Stomach tumors were recorded with an estimate of size, and many specimens were preserved for histological examination. Classification of mice as with or without a gastric tumor was based entirely on gross observations, the morphological variations not being considered.

OBSERVATIONS

In the breeder group (192 males, 292 females) the median age at death is the same in both sexes—about 535 days, or nearly a year and a half. In nonbreeding males (413 mice) the median is 340 days,

while in nonbreeding females (391 mice) the median is 392 days. The sex difference appears to have been due to a tendency to kill males first, although there was no intent to produce such a result, and it has no significant effect on the conclusions.

The incidence of stomach tumors at successive ages among mice of the four groups is presented in Chart 1. A strikingly lower frequency is shown throughout by the breeder females, while the other three groupings overlap or differ little among themselves up to the age of 400 days. After 400 days a moderate sex difference is also apparent in the nonbreeders.

Stomach tumors were rarely found in the mice that died when less than 6 months of age, but the frequency rapidly rises to a plateau by the age of about 9 months, except in the breeding females, where a rise is not obvious until the age of about 1 year. In the oldest age groups there is a slight reduction in gastric tumor frequency among breeders of both sexes.

An attempt has been made to analyze the correlation of tumor size and age at death. The data for nonbreeders afford the most satisfactory basis. Tumor size was determined for the solid part, cysts being ignored for this purpose. The estimates are admittedly rough but on the whole fairly reliable. The correlation is positive but low; many old mice had small tumors (about 2 or 3 mm. thick), while a few mice already had large tumors (a centimeter or more in diameter) at the age of 9 months (Chart 2). The median age for mice having the smallest tumors is about 340 days, while for mice having the largest tumors the median age is 400 days. Similarly, the median sizes of gastric tumors for mice less than a year old is in the small or medium-small range, while for mice over 1 year old the median tumor size is consistently greater.

There is some indication of a relation between the tumor and general health. In the nonbreeders, 74 mice *died* at an age of 200 days or older. Of these, only about 25 per cent had gastric tumors, a much lower incidence than for the group as a whole. In castrated Br-S mice of both sexes (6), increased incidence of gastric tumors has been reported and also a greater incidence of gastritis. However, the health of these mice has seemed good, and in mice of the Br-S strain it has been commonly noted that large gastric tumors tend to occur in large fat mice.

Genetic linkage tests.—An outcross has been undertaken to further our knowledge of the genetic basis of gastric lesions in mice of the Br-S strain. In a previous study, Strong (7) found that a majority of F_1 mice from an outcross of Br-S mice (treated with methylcholanthrene) to C57 blacks had gastric tumors, and in the F_2 generation close association of gastric tumor occurrence with the gene for brown coat color was observed. The conclusion drawn was that a single dominant gene was responsible for gastric tumors and that it was on the same chromosome with brown.

The mice in the study by Strong (7) were all injected with methylcholanthrene, while in the present test none were subjected to this carcinogen. The genetic formula for the Br-S mice is $\frac{abG}{abG}$, a being the nonagouti pattern; b, brown; and G, the presumed dominant gene for gastric tumors. The division line separates genes of maternal and of paternal derivation. A multiple linkage test was arranged as follows. A stock of mice related slightly to Br-S was developed with the formula $\frac{Wp\ \text{Wavy}\ se}{+p\ +\ +}$. This stock derived W (mutant dominant spotting) from strain N, where it had appeared as a mutation following methylcholanthrene injections. The heterozygote shows whitening effects in combination with other spotting factors but is slightly different from ordinary W when on a wild-type background, since the former produces hardly any white areas but, rather, scattered white hairs and a lightening of the yellow band in the hair. The homozygote, like that of ordinary W, is very anemic, white, and lives only a few days after birth. This mutant was combined with p (pink eye), another methylcholanthrene-induced mutation (recessive) in the CHI strain. Wavy, another dominant mutation, occurred spontaneously in strain JK. Whether it is the same as previously known wavy-1 or wavy-2 of other laboratories is not known. The gene se (short ear) was characteristic of the JK strain. The resulting stock was predominantly of CHI origin, since back-crosses to that strain were made. No stomach tumors have been observed in mice of CHI strain.

Reciprocal crosses of Br-S with this stock were made, and multiple-heterozygote F_1 mice were selected—chiefly males. These were immediately mated with JK females, a multiple recessive type. The formulation of the cross is as follows:

$$\frac{W\ p\ \text{Wavy}\ se+\ +\ +}{+\ +\ \ +\ \ +\ a\ b\ G} \times \frac{+p+se\ \ a\ b+}{+p+se\ \ a\ b+}.$$

As it turned out, most of the selected multiple-heterozygote F_1 mice did not get the se gene, so that linkage data with this gene are limited.

In addition to the above test crosses, a few mice were obtained in another test. A stock with wavy hair and d (dilution) was made up from a cross of wavy JK × C_{12}I, with selection in F_2. A male wavy dilute was next mated with Br-S females, and wavy offspring were selected (three females). These

COMPARISON OF GASTRIC TUMOR INCIDENCE IN FOUR GROUPS OF UNTREATED BrS
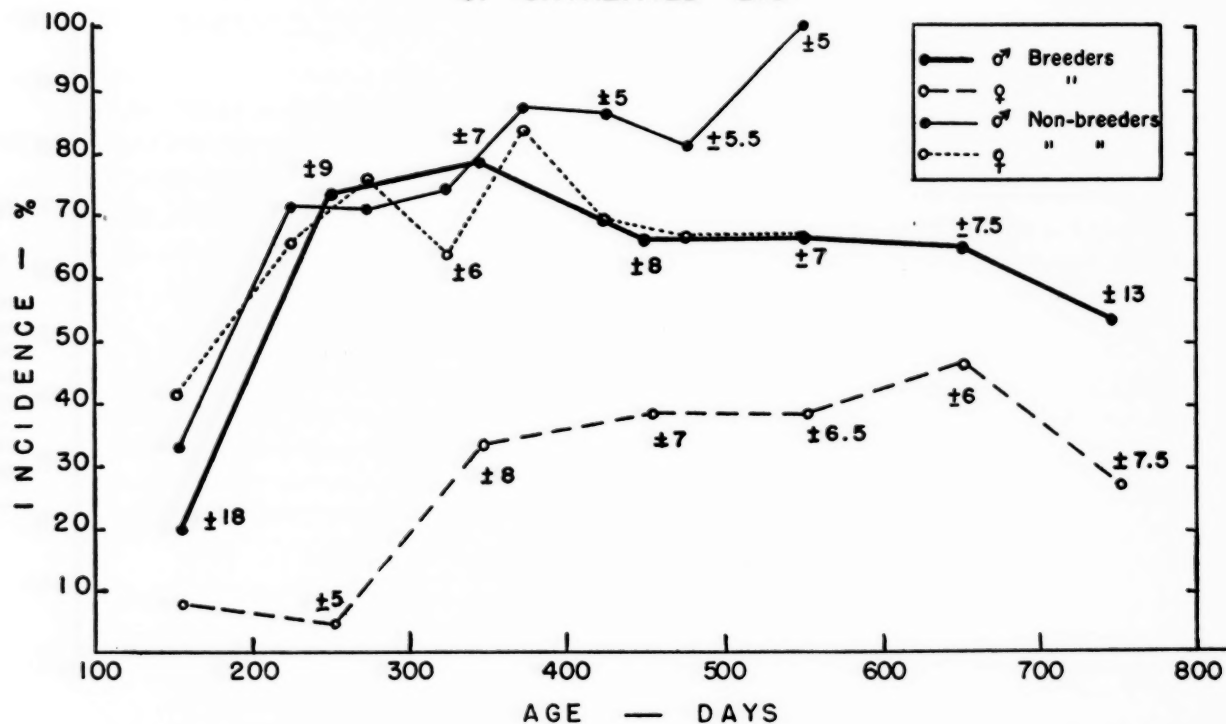
CHART 1.—Incidence of gastric tumors in mice of Br-S untreated strain according to sex, age, and breeding history. Groupings for age are by 100-day intervals or 50 days (non-breeders). Standard errors of the percentage incidence are noted for several points.
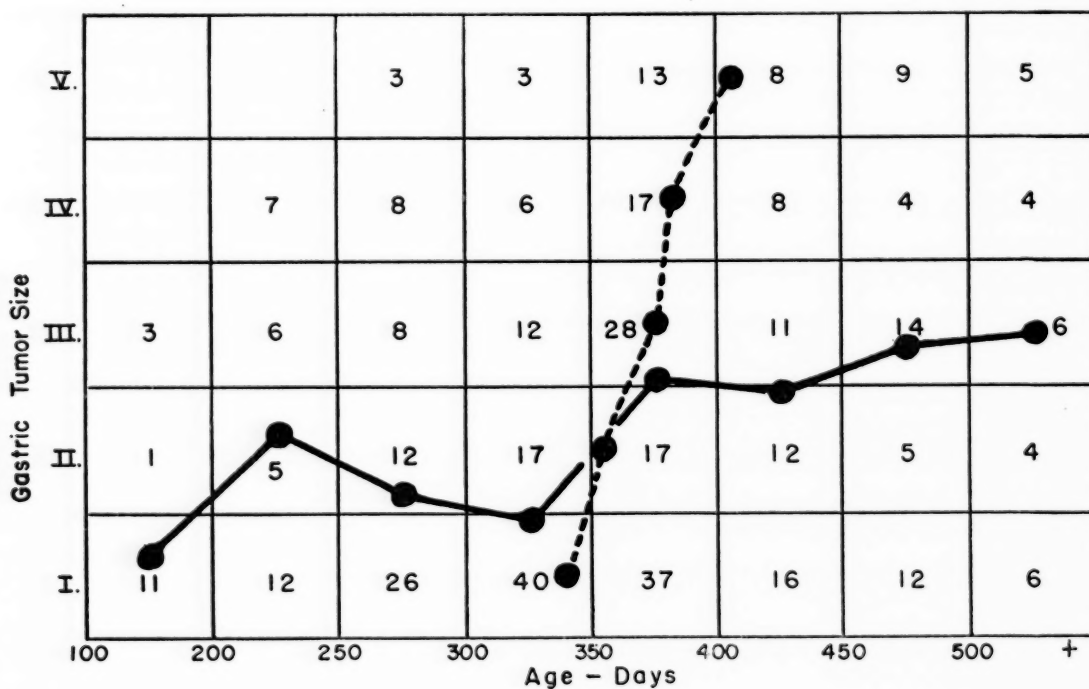
CHART 2.—A correlation table for age at necropsy and size of gastric tumor in nonbreeders of strain Br-S (untreated). The sexes have been combined. I, II, III, IV, and V are categories of tumor size from small to large. The numbers in the squares are frequencies. The regression lines are formed by connecting the medians of the rows and columns.

were then mated with a male of strain I. The formulation of this cross is as follows:

$$\text{Wavy } \frac{d + + + + +}{+ \quad + a \ b \ G + +} \times \frac{+ \ d \ a \ b + p \ s}{+ \ d \ a \ b + p \ s}.$$

As far as possible, the data from this test have been added in with the previous data. The genes *se* and *d* are closely linked so that their possible linkage relations to the gastric tumor factor should be almost identical and may be added together.

Except for the parental mice, which were breeders, practically all the animals were segregated as to sex and were kept in groups of six or fewer per box. A few escaped or died of other causes, but, out of 285 animals weaned, 251 were usable for necropsy at the required age. No mouse was included that died at 1 year or less of age, and in most cases they were killed at the age of 18 months. It is felt, therefore, that plenty of time had been afforded for tumors to develop.

The only difficulty in classification was with *W*. Accuracy in certain combinations is very high, while in combinations with *p* accuracy is poor, a number of these mice being unclassifiable for *W*. These dubious cases were arbitrarily thrown into one or the other category. One may assume about 10 per cent error.

The observed categories are given in Table 1. No definite evidence of linkage is seen in any test.

TABLE 1

LINKAGE TEST DATA INVOLVING
G (GASTRIC TUMOR FACTOR)

TEST BETWEEN	NONCROSS-OVERS G	not G	CROSS-OVERS G	not G
G and *a*	27	84	38	102
G " *b*	29	95	36	91
G " *p*	40	89	25	97
G " *W*	37	83	24	85
G " Wavy	38	93	27	93
G " *se* or *d*	9	13	8	10
Totals	180	457	158	478
Per cent	14.1	35.9	12.4	37.6
	50 per cent		50 per cent	

Obviously, the proportion of animals with gastric tumors is considerably below the theoretical expectation of 50 per cent (segregation of a single dominant gene), so that about a quarter of the animals was presumably misclassified as normal. Such an error is not serious for the purposes of the linkage tests, however, as the errors in the crossover group and the noncross-over group are simply exchanged and do not greatly affect the final percentage of crossing over.

The "penetrance" of gastric tumor (=percentage of animals that show the trait) is roughly about 70 per cent in the Br-S strain after 200 days of age (if we assume that all the mice were potentially

tumor-producers). In the F_1 generation from ♀ Br-S × ♂ W Wavy *pp* or wavy *dd* there were eight mice, of which three had gastric tumors (one of three females and two of five males). In the reciprocal cross of sixteen mice there were four cases of gastric tumor (two of five females and two of eleven males). On genetic grounds we should expect nearly as high an incidence here as in Br-S mice, but it is little over a third as high. In the linkage-test mice the total incidence is 26.5 per cent, while we might expect a maximum of 50 per cent. A breakdown of this generation, according to whether the parental F_1 mouse had gastric tumor or not, is presented in Table 2; in the former case,

TABLE 2

FREQUENCIES OF GASTRIC TUMORS IN THE LINKAGE TEST
ACCORDING TO THE F_1 INDIVIDUALS FROM WHICH
THE DESCENDANTS CAME

F_1	OFFSPRING ♂ Normal	♀	♂ Tumor	♀	PER CENT TUMOR
♂ A*	8	8	4		20
♂ B*	27	41	14	11	27
♂ C	10	9	4	2	24
♂ D	14	10	1	1	8
♂ E	7	5	1	3	25
♂ F	9	14	5	1	21
♂ G*	10	6	8	5	45
♂ H	1	2		2	40
♀ *		3			
♀	3	4	3		30

* With gastric tumor at necropsy.

29 per cent developed tumors, and in the latter, 21 per cent. The difference is not great and amounts to less than twice the standard error.

Tumor sizes in the linkage-test mice were, in general, small to medium; the incidence of large examples was much lower than in the Br-S mice.

The relation of gastric tumor to sex in the linkage-test mice is of interest. Of the 251 mice, 129 were males and 122 females, but of the 65 with the tumor, 41 were males (63.1 per cent). The sex difference here is significant. It will be noted that a similar deviation was true of the Br-S nonbreeders after the age of 400 days (Chart 1).

The relation of body weight to gastric tumor incidence was taken up more carefully in the linkage-test population. Statistical data are shown in Chart 3. In spite of similar incidence of tumors, the F_1 mice were markedly larger than the later generation. No weight difference of any significance could be found between mice with or without a gastric tumor.

DISCUSSION

In the study of Smith and Strong (6) it was observed that only 6 of 50 intact males autopsied between the ages of 200 and 600 days had gastric tumors, and of 42 intact females (nonbreeders)

seven had tumors in the same age range. These incidences are strikingly lower than those in the present study, and they require comment. The stocks used were slightly different, to be sure, but both were Br-S, and incidences in methylcholanthrene-treated Br-S mice have generally been high (excluding mice which died early of a local tumor). The chief difference between the conditions in the experiment of Smith and Strong (6) and those of the present study appears to be diet. In the former case Purina Laboratory Chow was used, while in our present case the staple food was Nurishmix (Pratt Food Company, Philadelphia), with some grain, bread, and milk supplement. A

cludes early mortality and lowered body weight. Large, fat mice do not necessarily show greater incidence of the tumor, but its size tends to be greater.

The most striking positive conclusion in this study is that breeding results in a greatly lowered incidence of gastric tumors in *females* of the Br-S strain. Presumably some factor or factors associated with gestation and/or lactation has inhibited the growth of the tumor; the hormones progesterone and prolactin are peculiar to breeding females. It is interesting to note, however, that breeding males show a generally lower incidence, after the age of 400 days, than that of the non-
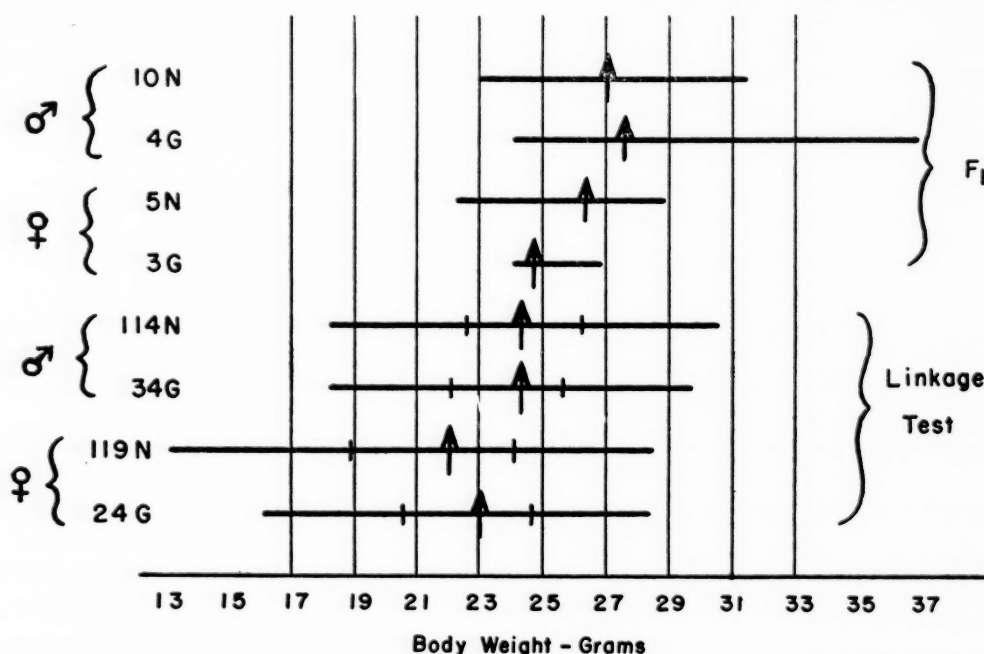

CHART 3.—Frequency distributions of body weights for mice in the linkage test, with gastric tumor (*G*) or normal (*N*). The horizontal lines indicate range, the arrows indicate medians, and the cross bars indicate quartiles.

relation of tumor incidence or development to gastric activity is indicated in the experiments of Bagshaw (2) in which vagotomy of young Br-S mice resulted in decreased incidence of the tumors. Andervont (1) noted a greater development of gastric lesions in mice of the I strain receiving a diet containing much roughage.

The association of age and tumor size suggests that these tumors may develop rather rapidly for a while, probably at about 8–10 months of age (except in female breeders), and then become quiescent. This interpretation differs somewhat from the suggestion by Smith and Strong (6) that the "lesion grows slowly."

Poor health is more likely to be associated with low incidence of gastric tumors and smaller size of those occurring. Poor health (as used here) in-

breeding males. The difference seems significant, although the number of nonbreeder males at those ages is rather small; the same tendency is found in the linkage-test data but not in the data of Smith and Strong (6). The sex differences offer an analogy to the sex difference in human gastric ulcers (5), as well as that of gastric carcinoma.

Differences in health seem unlikely as an explanation of the low tumor incidence in female breeders. The gastric tumor incidence in these females does not rise until the age of about a year. This age is a rather common division point between normal reproduction and cessation or a marked decline indicative of senility. The reciprocal relation between reproduction and gastric tumor incidence does not continue, however; one might expect the incidence in completely sterile

senile females to rise toward 100 per cent, but instead it is less than 50 per cent, even in the oldest group. Another point to consider is the tendency for nonbreeder (virgin) females to remain at a slightly lower weight level than breeder females.

The castrated mice in the experiment of Smith and Strong (6) developed gastric tumors much more often than the intact controls: 36 out of 71 males and 28 out of 68 females (all over 200 days old). It is possible that an incidence of 100 per cent may be achieved with castrated mice on Nurishmix diet.

The genetic data in the present study indicate that the gene (or genes) involved in susceptibility to this type of gastric tumor is dominant, but with penetrance of varying value—usually less than 70 per cent. No linkage was revealed in these tests, in contrast to results obtained by Strong (7) in a test with outcrosses to strain C57, where close linkage with *b* was found. Whether or not the methylcholanthrene treatment in that test was a differentiating factor is unknown and deserves further study.

The sex differences here observed give no support to an interpretation of sex-linked inheritance of gastric tumor. A physiological explanation seems much more likely.

The problems of maternal influence, as in litter-seriation effects, on gastric tumor incidence remain to be explored. Litter seriation has been ignored in the present study, since most of the Br-S mice were born in very early litters, and those in the linkage test were not followed.

SUMMARY

The incidence of adenomatous neoplasia of the stomach in mice of a control sub-line of the inbred Br-S strain is reported, with reference to age, sex, and reproductive activity. The tumors rarely appear before the age of 6 months, but incidence in nonbreeders and in male breeders reaches a maximum at about a year. Breeder females show a marked lag and a much lower incidence at all ages. Size of tumor was somewhat correlated with age, although tumors of all sizes occurred at any age after about 10 months.

An outcross of this strain was made to obtain genetic linkage tests involving the genes *a*, *b*, *d*, and *se*, *p*, *W*, and a wavy-hair mutant. The incidence of gastric tumors was uniform in all classes, so that no linkage was indicated. This result contrasts with an earlier report of close linkage of *b* with the gastric tumor factor in tests with mice of the methylcholanthrene-treated line. Dominant inheritance with incomplete penetrance is indicated. In the present linkage-test population no relation of tumor incidence to body weight could be detected.

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Studies on the Formation of Protein-bound Derivatives of 3,4-Benzpyrene in the Epidermal Fraction of Mouse Skin*

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INTRODUCTION

The general metabolism of the carcinogenic hydrocarbons has been studied intensively by a number of workers (2, 4, 5). However, the reactions occurring in the tissues susceptible to their carcinogenic activity have received relatively little attention, although such studies should be of particular value in determining the mechanism by which the tumors are initiated. The late F. Weigert and his associates (15, 17–19) have shown the presence of certain derivatives of 3,4 benzpyrene in sites where it was injected subcutaneously or applied to the skin of mice. Most of the metabolites in the skin were present in a form which could not be extracted prior to alkaline hydrolysis of the tissue. Recently, we have observed protein-bound derivatives of 3,4-benzpyrene in the epidermis of mice following topical application of the hydrocarbon (7). Studies on the occurrence and properties of these derivatives form the subject of this paper. The search for these derivatives was undertaken, since the carcinogenic aminoazo dyes (or azo metabolites thereof) have been shown to combine with certain proteins of the liver (the susceptible tissue) in the rat. This reaction appears to have causal significance in liver carcinogenesis (8, 9).

METHODS

Purification of 3,4-benzpyrene.—All the 3,4-benzpyrene (Hoffman LaRoche, Inc.) was purified in a dark room by chromatography on activated alumina (10) in 20- to 45-mg. lots. Either pure petroleum ether (Skelly Solve B, b.p. 66°–68° C.) or petroleum ether containing up to 2 per cent of benzene (depending on the activity of the alumina) was used as solvent and eluant. After elution the solvent was removed *in vacuo* (water bath temperature less than 45° C.), and the 3,4-benzpyrene was dissolved in ethanol and precipitated by the addition of water. This procedure removed two fluorescent bands, one moving at a faster and one at a slower rate than the main band of 3,4-benzpyrene; the resulting product appeared homogeneous when rechromatographed on alumina. While it was necessary to follow the band by its fluorescence under ultraviolet light, extreme care was used to prevent photo-oxidation of the hydrocarbon. In particular, the chromatographic column was covered and was viewed with ultraviolet light only momentarily through a vertical slit a few mm. wide. Furthermore, throughout the study all solutions containing the carcinogen or its derivatives were always shielded from light by black cloth or paper covers.

Animal experiments.—Female albino mice[1] about 2–4 months of age were used throughout. The mice were kept in screen-bottom cages in groups of three to seven and were fed a stock diet[2] and water *ad libitum*. The hair was removed from a 12–15 sq. cm. area on the backs of the mice with a surgical clipper prior to each experiment, and individual mice were reclipped when necessary during the experiments. In general, 4–6 drops of either benzene or a 0.2 per cent solution of 3,4-benzpyrene in benzene were applied as uniformly as possible to the clipped area of each mouse. This corresponded to 200–250 μg. of hydrocarbon per application, and the average dose was recorded for each experiment. Where large quantities of epidermal protein were needed for chemical studies, 50–75 mice were treated daily for 5 days with either benzene or a 0.2 per cent solution of 3,4-benzpyrene, and the mice were killed on the sixth day. For studies on the rate of accumulation of the derivatives under different conditions, groups of

[1] Purchased from Arthur Sutter, Springfield, Mo.

[2] Friskie Dog Biscuits, purchased from the Carnation Co., Milwaukee, Wis.

mice were treated with benzene or the hydrocarbon solution, and groups of three to six mice were killed at the intervals indicated for each experiment.

In all cases the mice were killed with ether, and the treated area of the skin was excised. The skins were immersed in N/3 ammonia solution (1) for 20–30 minutes, and the epidermal area was then scraped away from the dermal portion with a scalpel. Both portions were collected in ice-cold beakers protected from the light. The dermal fraction was ground through the fine blades of a household meat grinder, and then both fractions were ground separately in a Waring Blendor in an 85 per cent ethanol solution containing 10 per cent of trichloroacetic acid. The suspension was centrifuged, and the precipitate was washed 3 times with ethanol. For the preparation of protein from the internal organs, the tissues were homogenized in water in the Waring Blendor, and aqueous trichloroacetic acid was added to give a final concentration of 9 per cent. The proteins were sedimented by centrifugation and then washed successively once with a pH 5, 1 M acetate buffer and twice with ethanol. Finally, all protein fractions were wrapped individually in filter paper, extracted at 60° C. for 48 hours with ethanol in a Soxhlet apparatus, and dried *in vacuo* over sulfuric acid. The resultant crude preparations thus consisted chiefly of a mixture of protein and nucleoprotein.

All the solvents used for purifying and applying the hydrocarbon, washing and extracting the protein preparations, and carrying out the analytical work were redistilled from an all-glass apparatus and were essentially free of fluorescent materials. All the glassware for these experiments was cleaned with sulfuric acid–sodium dichromate solution maintained at 50°–60° C. Contact with corks, rubber stoppers, and stopcock grease was scrupulously avoided.

Analytical procedures.—For analysis, 25 mg. of the crude protein preparation and 2 ml. of ethanol, 5 ml. of 4 N potassium hydroxide, 5 ml. of toluene, and about 1.6 gm. of zinc dust were heated in a 1 × 8-inch test tube at reflux on a sand bath for 2 hours. After cooling, the hydrolyzate was extracted 3 times with 15-ml. aliquots of benzene. The zinc dust was then packed by light centrifugation, the aqueous layer was decanted into a clean test tube, and the zinc was washed with 2 ml. of 3 N potassium hydroxide in 30 per cent ethanol. The combined aqueous layer and washings were acidified by the addition of 5 ml. of 7 N HCl, and the solution was extracted 3 times with 15-ml. aliquots of benzene. A motor-driven assembly fitted with ten spiral glass stirrers was used for the extractions, while a suction device consisting of a capillary tube equipped with a standard taper glass joint was used to transfer the benzene extracts from the test tubes to 100-ml. graduated cylinders fitted with complementary joints. The benzene extracts from the alkaline and from the acid solutions, i.e., the neutral and acidic fractions, respectively (see below), were each diluted to 70 ml. and dried with sodium sulfate, and their fluorescent intensities were determined in a Coleman fluorophotometer equipped with the B_1-B_2 filters. All the samples were read against a standard quinine sulfate solution, and, since the identities of the derivatives are not known, all the results are expressed in terms of "fluorescence units." These units are identical to the galvanometer readings with this instrument. Duplicate analyses for each fraction usually agreed within 10 per cent.

Hydrolysis of the protein with 4 N KOH under these conditions liberated essentially all the fluorescent derivatives within 20 minutes, and no destruction was found within hydrolysis periods up to 4 hours. The 2-hour hydrolysis was used routinely, since troublesome emulsions formed upon extraction of samples hydrolyzed for shorter periods. Rehydrolysis of the neutral derivative under these conditions gave quantitative recoveries, while only 70–90 per cent of the acidic derivative was recovered. Neither derivative gave rise to appreciable levels of the other on rehydrolysis. When 20- to 50-mg. samples of the protein were hydrolyzed and extracted under the conditions listed above for 25-mg. samples, the amounts of each fluorescent derivative were approximately in proportion to the amount of protein used.

The reductive hydrolysis was suggested by Martin's modification (6) of the Clemmensen reduction, in which anthracene was obtained in 93 and 80 per cent yields by heating anthrone and anthraquinone, respectively, in the presence of alkali, toluene, and zinc dust activated with copper sulfate. The importance of the zinc dust in the estimation of the fluorescent derivatives of 3,4-benzpyrene is shown in Table 1. When the zinc dust was omitted, very little or none of the neutral derivative could be extracted, while the fluorescence of the acidic extracts was usually unchanged but occasionally increased up to 25 per cent. The extracts from the alkaline hydrolyzates had a maximum fluorescence when 0.8 or 1.6 gm. of zinc dust were added per tube. Similar results were also obtained with as little as 0.1 gm. of zinc dust which had been activated by suspension for 15 minutes in 15 ml. of a 0.2 per cent solution of copper sulfate;

the copper sulfate was then decanted, and the moist zinc was used immediately. Small amounts of the copper sulfate solution in the absence of zinc did not give rise to the fluorescent neutral derivative. Although the addition of toluene to the hydrolysis mixture was not essential, the analyses for the neutral component appeared to be more reproducible when it was used. The nature of the reactions occurring under these reducing conditions has not been investigated. However, alkaline hydrolysis in the presence of sodium hydrosulfite did not yield a neutral fluorescent derivative and reduced the yield of the acidic fluorescent derivative.

TABLE 1

VARIATIONS IN THE LEVELS OF THE NEUTRAL AND ACIDIC DERIVATIVES OF 3,4-BENZPYRENE FROM 25 MG. OF EPIDERMAL PROTEIN AS A FUNCTION OF THE AMOUNT OF ZINC DUST USED IN THE HYDROLYSIS

Epidermal protein sample	Zinc dust (gm/tube)	Fluorescence of neutral derivative (units)	Fluorescence of acidic derivative (units)
Benzene-treated	0	4	2
	1.6	3	2
	0.8 (activated*)	3	2
3,4-Benzpyrene-treated	0	6	26
	0.1	16	26
	0.8	34	26
	1.6	36	24
	0.1 (activated)	34	24
	0.8 (activated)	33	24

* The zinc dust was stirred in 15 ml. of a 0.2 per cent solution of copper sulfate for 15 min. The copper sulfate solution was decanted before use; small quantities of the copper sulfate solution had no effect on the liberation of these compounds.

RESULTS

Properties of the fluorescent derivatives.—For studies on the properties of the fluorescent derivatives, 1- to 2-gm. batches of epidermal protein were prepared, as described above, from groups of 50–75 mice which had been treated topically with either benzene or a benzene solution containing 0.2 per cent of 3,4-benzpyrene on 5 successive days. The preparations from the hydrocarbon-treated mice contained a high level of both fluorescent derivatives, while those from the benzene-treated mice had very little fluorescence and were used as control material. The solutions of the hydrocarbon derivatives were obtained by the procedures described under "Methods."

The hydrocarbon derivatives, obtained when the hydrolysis of the protein was carried out in the presence of zinc dust, had similar fluorescent properties but different solubilities. The *neutral* derivative could be extracted from alkaline, neutral, or acidic solutions with benzene, but for convenience was routinely extracted from the alkaline hydrolyzate. The compound appeared to have some polar character, since, on shaking a benzene solution of the derivative with an aqueous solution, the compound distributed itself between the two phases. The *acidic* derivative could not be extracted with benzene from alkaline solutions, but was extracted gradually from neutral solutions and readily from acidic solutions. This derivative was quantitatively removed from a benzene solution by extraction with a small amount of alkali.

Like 3,4-benzpyrene, both the neutral and acidic derivatives had a blue fluorescence upon irradiation with the 360 mμ band from a mercury lamp; the fluorescent light was transmitted by a filter (Coleman B$_2$) with maximum transmission at 440 mμ (range, 420–480 mμ). However, the fluorescence of the derivatives was much less readily quenched by oxygen than that of 3,4-benzpyrene (12). Thus, when a benzene solution of 3,4-benzpyrene was saturated with nitrogen by bubbling the gas through a fine capillary, the fluorescence of the solution was 9 times greater than when the same solution was saturated with oxygen. However, when benzene solutions of the neutral and acidic derivatives were similarly gassed, their fluorescent intensities were only 2.3 and 2.5 times greater when the solution was saturated with nitrogen instead of oxygen. When 3,4-benzpyrene was added to either of these solutions, its fluorescence was still 9 times greater when the solution was saturated with nitrogen rather than oxygen.

Evidence that the derivatives are combined chemically with the protein.—Most of the chemical studies reported in this paper were carried out to determine whether the fluorescent derivatives of 3,4-benzpyrene were combined chemically with the protein, adsorbed on the protein, or combined with a contaminant in the crude protein preparation. For these studies 1- to 2-gm. batches of hot-ethanol-extracted crude epidermal protein were prepared, by the methods described above, from mice treated either with benzene or with a benzene solution containing 0.2 per cent of 3,4-benzpyrene on 5 successive days. After the treatments described, in each case the preparations were analyzed by the routine analytical procedure.

In one experiment 60-mg. samples of epidermal protein were extracted with three 30-ml. changes of boiling solvents over a 10-hr. period. If the derivatives had been only adsorbed to the protein, significant losses would have been expected, but the content of the 3,4-benzpyrene derivatives was not altered when the boiling solvent was ethanol, ethyl ether (peroxide-free), or benzene (Table 2).

Similar treatment with boiling pyridine caused a 20 per cent loss of the fluorescent derivatives, but this decrease may have been caused by the basic properties of the pyridine rather than by a simple extraction.

Studies on the rate of liberation of the fluorescent derivatives also indicated that the neutral derivative, at least, was held by a bond stronger than that of the usual adsorption complex. Thus, when the hydrolysis was carried out with ethanolic 4 N KOH at boiling, the cleavage of both fluorescent derivatives was complete within 20 minutes. However, when the hydrolysis was carried out in a 70° C. oil bath, only 40–50 per cent of the neutral derivative was liberated within 20 minutes, al-

TABLE 2

LEVELS OF THE BOUND DERIVATIVES OF 3,4-BENZPYRENE
IN EPIDERMAL PROTEIN FOLLOWING EXTRAC-
TION WITH BOILING SOLVENTS*
(Expressed as fluorescence units per
25 milligrams of protein)

Sample	Solvent	Neutral derivative	Acidic derivative
3,4-Benzpyrene-treated	None	21	22
	Benzene	23	21
	Ethanol	21	22
	Ethyl ether†	23	21
	Pyridine	16	16
Benzene-treated	None	4	1
	Benzene	4	1
	Ethanol	4	2
	Ethyl ether	4	1
	Pyridine	4	2

*Sixty mg. of protein extracted with three 30-ml. changes of boiling solvent over a 10-hr. period.
† Peroxide-free.

though all the protein was in solution. Maximum cleavage was attained in 1 hour, and the fluorescent intensity of the extract was the same as that obtained by hydrolysis at 85° C. The rate of hydrolysis also decreased when lower concentrations of KOH were used. Thus, with 1 N KOH and in an 80° C. bath only 50 and 60 per cent as much of the neutral derivative could be extracted after 20- and 60-minute hydrolyses, respectively, as could be extracted after a 3- to 5-hour hydrolysis. However, when lower concentrations of alkali were used for hydrolysis, the maximum fluorescent intensity of the neutral extract, even after prolonged hydrolysis, was always less than when 4 N KOH was used. The fluorescent intensity and the rate of liberation of the acidic derivative appeared to be relatively unaffected by the concentration of alkali and the time of hydrolysis.

For a more rigorous test of chemical combination, 100- to 200-mg. samples of epidermal protein from either benzene- or 3,4-benzpyrene-treated mice were suspended in 14 ml. of a 1:2.5 mixture

of ethanol and 0.1 N KOH/100 mg of sample and finely dispersed with a loose Potter-Elvehjem homogenizer. Each preparation was then transferred to a 125-ml. flask and incubated as described below. At the end of the incubation period the suspension was cooled quickly, and the residual protein was sedimented by centrifugation. The clear supernatant solution was decanted carefully, the soluble protein was precipitated by the addition of trichloroacetic acid to a final concentration of 10 per cent, and the precipitated protein was separated by centrifugation. Both the residual protein and the protein precipitated with trichloroacetic acid were washed once with a pH 5, 1 M acetate buffer and twice with ethanol, extracted for 48 hours with ethanol at 60° C. in a Soxhlet apparatus, dried *in vacuo*, and analyzed. All transfers were made quantitatively so that the recovery of protein could be determined.

Essentially the same results were obtained whether the protein was incubated in a 55° C. bath for 18 minutes or in a 45° C. bath for 30 minutes (Table 3). In the latter case, the reprecipitated proteins had the same concentration of the acidic derivative as the original protein but only 26 per cent as high a concentration of the neutral derivative. The concentrations of the neutral and acidic derivatives in the residual protein were 41 and 56 per cent, respectively, of those in the original protein. The over-all recoveries of the hydrocarbon derivatives in the two fractions were 31 per cent for the neutral derivative and 53 per cent for the acidic derivative. These recoveries were increased to 60 and 90 per cent, respectively, by analysis of the combined aqueous and ethanolic solutions obtained in the preparation and washing of the protein fractions. The solubilized derivatives were about equally distributed between the aqueous and ethanolic solutions, and about half of those in the aqueous solutions could not be extracted prior to alkaline hydrolysis. Although some of the derivatives of 3,4-benzpyrene were solubilized by these treatments, the retention of very significant quantities by the reprecipitated proteins is strong evidence in favor of a chemical linkage between derivatives of the carcinogen and the epidermal protein. It should be noted that these conditions were drastic enough to degrade 20–25 per cent of the protein to fragments no longer precipitable with trichloroacetic acid, and there is suggestive evidence that the fluorescent derivatives were attached to some of these fragments.

With more rigorous conditions the yield of fluorescent derivatives remaining on the protein decreased, but even after 60 minutes in an 80° C. bath the concentration of the acidic derivatives on

the reprecipitated proteins was 59 per cent of that in the original protein (Table 3). However, the neutral derivative could just be detected in the reprecipitated protein, and neither fraction was found in the residual protein. In this case 53 per cent of the protein was degraded to a size which was no longer precipitated with 10 per cent trichloroacetic acid. When an 80° C. bath was used for 30 minutes, the concentration of the acidic derivative on the reprecipitated protein was 83 per cent of that on the original sample. Although the levels were low, the neutral derivative could be detected in the reprecipitated protein, and both

Content of protein-bound derivatives of 3,4-benzpyrene in various tissues.—To determine the distribution of the protein-bound derivatives of 3,4-benzpyrene in various tissues, a group of ten mice was given daily applications of 220 μg. of 3,4-benzpyrene in benzene solution on the skin of the back for 5 days, and a control group received similar applications of benzene alone. The mice were killed 20 hours after the last application, and protein preparations were made from various tissues. On analysis the epidermal protein from the treated area contained 35 and 30 units, respectively, of the neutral and acidic derivatives per 25 mg., while

TABLE 3

LEVELS OF THE BOUND DERIVATIVES OF 3,4-BENZPYRENE IN EPIDERMAL PROTEIN
FOLLOWING SOLUTION AND REPRECIPITATION*
(Expressed as fluorescence units per 25 milligrams of protein)

SAMPLE	30 MIN. AT 45° C.			18 MIN. AT 55° C.			30 MIN. AT 80° C.			60 MIN. AT 80° C.		
	Wt. (mg.)	Neutral deriv.	Acidic deriv.	Wt. (mg.)	Neutral deriv.	Acidic deriv.	Wt. (mg.)	Neutral deriv.	Acidic deriv.	Wt. (mg.)	Neutral deriv.	Acidic deriv.
Original, BP-treated†	200	39	25	150	40	29	150	33	23	100	20	17
Residue, BP-treated	131	16	14	75	14	12	58	3	6	27	2	3
Reprecipitated, BP-treated	31	10	25	36	10	31	40	4	19	20	2	10
Original, benzene-treated	200	2	1	150	2	1	150	1	1	100	1	1
Residue, benzene-treated	142	2	2	86	2	1	67	2	1	33	1	1
Reprecipitated, benzene-treated	16	2	1	24	2	1	32	2	1	17	1	1

* Fourteen ml. of a 1:2.5 mixture of ethanol and 0.1 N KOH were used for each 100 mg. of protein. Samples were incubated in 125-ml. Erlenmeyer flasks, and temperatures reported are for the water bath.

† BP = 3,4-benzpyrene.

types could be detected in the residual protein. Thirty-four per cent of the protein was not recovered in this experiment.

In all these experiments the total protein recovery was essentially identical for the epidermal proteins from mice treated with either benzene or 3,4-benzpyrene in benzene solution. However, the yield of reprecipitated protein was consistently greater and the amount of residual protein consistently less when the proteins from 3,4-benzpyrene-treated mice were used (Table 3).

Preliminary experiments with Schneider's hot trichloroacetic acid extraction of nucleic acids (16) indicated that the fluorescent derivatives were probably not attached to the nucleic acid fraction of the crude protein mixture. Although the extraction procedure solubilized about 30 per cent of the original crude protein so that considerable amounts of protein as well as nucleic acid were apparently degraded, the remaining extracted protein still contained about one-third and one-half as great concentrations of the neutral and acidic fluorescent compounds as the original protein.

neither derivative could be detected in the dermal protein from the treated skin or the proteins from untreated skin obtained from the same animals (Table 4). Furthermore, no protein-bound derivatives of the hydrocarbon were found in the proteins from the kidneys, hearts, lungs, intestines, or livers from these mice.

Rate of accumulation of protein-bound fluorescent derivatives in epidermis.—A single application of a 0.2 per cent solution of 3,4-benzpyrene in benzene (approximately 300 μg.) was sufficient to give rise to appreciable levels of the protein-bound derivatives (Chart 1). Within 3 hours after the application of the carcinogen, detectable levels had been formed, and maximum levels of 20 and 10 units, respectively, of the neutral and acidic derivatives per 25 milligrams of protein were found in epidermal samples taken at 24 hours. Thereafter, the amounts of both derivatives decreased slowly. Samples from mice killed at 4 and 7 days still contained approximately one-half and one-fourth, respectively, of the maximum levels. At 2 weeks, the derivatives could just be detected; no protein-

bound fluorescence was found in samples collected 3 or 6 weeks after the single application of the hydrocarbon.

Considerably higher levels of the protein-bound derivatives of 3,4-benzpyrene were obtained when the carcinogen was applied on several successive

TABLE 4

THE DISTRIBUTION OF PROTEIN-BOUND DERIVATIVES OF 3,4-BENZPYRENE IN THE MOUSE FOLLOWING APPLICA-TION OF THE CARCINOGEN TO THE SKIN ONCE DAILY FOR 5 DAYS*

(Expressed as fluorescence units per 25 milligrams of protein)

	NEUTRAL FRACTION FROM MICE TREATED WITH		ACIDIC FRACTION FROM MICE TREATED WITH	
TISSUE	Benzene (units)	3,4-Benzpy-rene (units)	Benzene (units)	3,4-Benzpy-rene (units)
Skin from back (treated area):				
Epidermis	3	35	2	30
Dermis	3	3	2	2
Skin from abdo-men (untreated area):				
Epidermis	3	4	2	4
Dermis	3	3	3	2
Kidney	5	4	3	3
Heart	3	3	3	3
Lung	3	4	3	3
Small intestine	3	3	2	3
Large intestine	3	2	1	2
Liver	4	3	1	2

* 0.2 per cent solution in benzene; total dose approximately 1.1 mg. per mouse.

days. With daily applications of 200 μg. of 3,4-benzpyrene, the levels rose rapidly following the first five applications and maintained a plateau at approximately 40 units of each derivative per 25 milligrams of protein from the fifth to the eighth application (Chart 2). When 300 μg. were applied daily, the curve rose more steeply and plateaued at approximately the same level after the third or fourth application. Experiments of longer duration were impractical, since exfoliation of some of the treated areas began to occur following the ninth or tenth application.

The levels of both the protein-bound fluorescent derivatives were greatly decreased when the mice were kept in bright light. In this study three groups of mice were treated similarly, except that the mice of one group were kept in darkness, those of another group were directly in front of an east window (in December), and those of the third were placed 15 inches below 40-watt incandescent lamps for 7 hrs/day. In the latter case, each cage of mice was centered below a different lamp. The animals were kept in cages made entirely of $\frac{1}{4}$-inch wire mesh, and only five mice were kept in each cage. Fifteen mice in each group received daily ap-

plications of 250 μg. of 3,4-benzpyrene on the skin of the back 2 hours before the end of the irradia-tion period, while five controls were treated only with benzene. Groups of five mice treated with the carcinogen were killed 24 hours after the first, third, and fifth applications; the control mice were killed after the fifth application. The levels of the neutral derivative in the epidermal protein from each of these groups are plotted in Chart 3. A similar plot for the acidic derivative is not given, since the shapes of the curves were very similar to those for the neutral derivative. After five applica-tions 25 mg. of epidermal protein from the mice kept in the dark contained 42 and 39 units, respec-tively, of the neutral and acidic derivatives. At the same time 25 mg. of epidermal protein from the mice irradiated by incandescent lamps contained only 18 and 19 units, respectively, of these deriva-tives, while only 5 units of each derivative were found in 25 mg. of epidermal protein from the mice maintained in the direct sunlight. In other experi-ments it was found that mice maintained in the diffuse light of the animal room had essentially the same levels of the fluorescent derivatives as those kept in darkness.

Attempts to produce the protein-bound derivatives in vitro.—A few preliminary studies were carried out to determine whether the protein-bound deriva-tives of 3,4-benzpyrene could be formed *in vitro*.
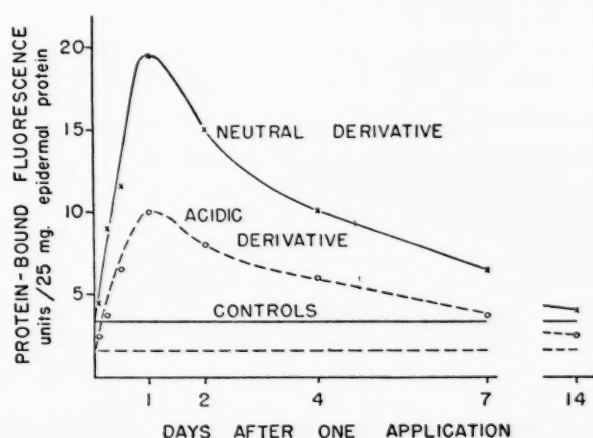

CHART 1.—Levels of protein-bound derivatives of 3,4-benz-pyrene in the epidermis of mice following a single topical appli-cation of 300 μg. of the carcinogen per mouse at zero time. The solid and broken lines at the bottom of the graph indicate the fluorescence in the neutral and acidic fractions from benzene-treated epidermis.

In one experiment the mice were killed, their hair clipped, and the skin from the back excised. The skins were pinned on a filter paper-covered board and kept at room temperature in a tightly covered glass dish. The filter paper was moistened with a small amount of 0.9 per cent saline solution con-

taining 0.5 per cent phenol. Twenty hours after a single application of 300 μg. of 3,4-benzpyrene in benzene solution, the epidermis was separated from the dermis of each skin, and the proteins were prepared from each fraction in the usual manner. No protein-bound fluorescent derivatives could be detected in the epidermis or dermis from the mice treated either with 3,4-benzpyrene or with benzene alone. Eviscerated dead mice were used in the second series. To reduce bacterial con-
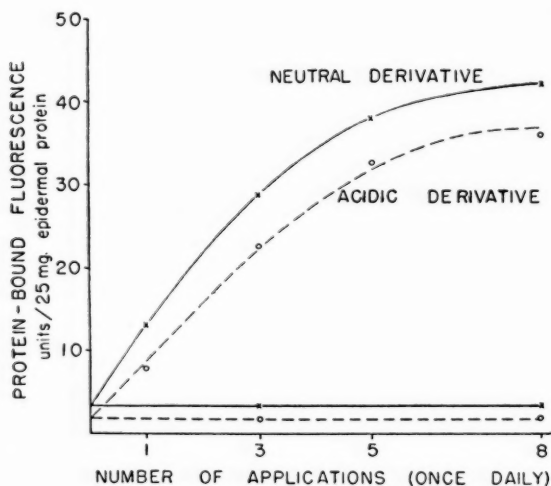

CHART 2.—Rate of accumulation of protein-bound derivatives of 3,4-benzpyrene in the epidermis of mice when 200 μg. of the hydrocarbon in benzene solution were applied daily for 1–8 days. The solid and broken lines at the bottom of the graph indicate the fluorescence in the neutral and acidic fractions from benzene-treated epidermis.

tamination, the inside of the body cavity was covered with a penicillin ointment, the cavity was filled with glass wool, and the opening was sutured and covered with the ointment. The mice were kept in tightly covered glass dishes as before, and given two applications of 300 μg. each of 3,4-benzpyrene. Twenty-four hours elapsed between treatments, and the mice were killed 20 hours after the second application. Groups of six such mice were kept in the dark or in bright sunlight, and no protein-bound fluorescent derivatives could be detected in the epidermis from either the hydrocarbon-treated or control mice. Both experiments were complicated by obvious contamination with microörganisms, but this appeared to be considerably less in the second series.

Analyses for protein-bound fluorescent derivatives following the application of croton oil or 20-methylcholanthrene.—As evidence that the fluorescent compounds were derived from 3,4-benzpyrene rather than being a nonspecific hyperplastic response, several mice were treated daily for 5 days with benzene solutions containing 0.5 per cent of croton oil, 0.2 per cent of 20-methylcholanthrene, or 0.2 per cent of 3,4-benzpyrene. Croton oil was used since it is noncarcinogenic but causes an intense hyperplastic response, while 20-methylcholanthrene is a more potent skin carcinogen and hyperplastic agent than 3,4-benzpyrene but emits only one-third to one-fourth as much fluorescent light as the latter carcinogen (11). On analysis both the neutral and acidic extracts from 25 mg. of the epidermal proteins from control and croton oil-treated mice contained about 2 fluorescence units, while the extracts from 3,4-benzpyrene-treated epidermis contained about 35 units each. The neutral and acidic extracts from 25 mg. of epidermal protein from the mice treated with 20-methylcholanthrene each contained 4 fluorescence units; a weak fluorescence might be expected in this case if protein-bound derivatives of 20-methylcholanthrene were formed in the treated epidermis.

DISCUSSION

Although final proof will rest with the characterization of the fluorescent compounds from mouse epidermis, the evidence presented here suggests
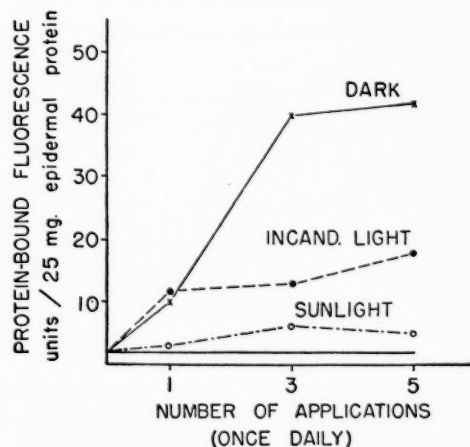
CHART 3.—Levels of the protein-bound neutral derivative of 3,4-benzpyrene in the epidermis of mice treated daily with 250 μg. of the hydrocarbon and maintained in darkness, in direct sunlight, or under incandescent lamps. The solid line at the bottom of the graph indicates the protein-bound fluorescence obtained from mice treated only with benzene.

strongly that the compounds are derivatives of 3,4-benzpyrene which were combined chemically *in vivo* with the epidermal protein. Their relationship to 3,4-benzpyrene is indicated by the rapid appearance of the derivatives after only one application of the carcinogen and by the lack of this response following the application of 20-methylcholanthrene or croton oil, which produce a similar or greater hyperplastic response but are much less fluorescent. The derivatives can be differentiated

from 3,4-benzpyrene by their different solubility properties, and, furthermore, the fluorescence of 3,4-benzpyrene solutions is quenched to a much greater extent by dissolved oxygen than the fluorescence of solutions of the derivatives. The evidence that the fluorescent derivatives are combined chemically with the epidermal protein rests in part on the isolation of a hot ethanol-extracted crude protein fraction from the epidermis prior to analysis. Further evidence was obtained by the lack of extraction of the derivatives from the protein by boiling solvents, and even stronger evidence was furnished by the finding of a high concentration of the acidic derivative and significant quantities of the neutral derivatives on protein which had been dissolved in weak alkali and reprecipitated with trichloroacetic acid. Further studies will be necessary to determine the relationship of these derivatives of 3,4-benzpyrene to the metabolites found by the British workers (15, 17–19) following the application of the carcinogen to mouse skin *in vivo* or *in vitro*. While they were unable to extract these metabolites from skin unless the tissue was first hydrolyzed with alkali, the nature of the tissue components with which the hydrocarbon was combined has not been reported.

Although the role of the 3,4-benzpyrene derivatives in the carcinogenic process cannot be properly assessed at this time, several observations suggest that there might be a causal relationship between the formation of these derivatives and the eventual appearance of tumors. First, following topical application of the carcinogen the protein-bound derivatives were found only in the epidermis of the treated area; this is the site at which tumors eventually arise. The derivatives were not found in the dermal portion of the treated skin, in the skin from untreated areas, or in any other tissue that was analyzed. Secondly, a single application of approximately 20 μg. of 3,4-benzpyrene per square centimeter of skin gave rise to very significant levels of the protein-bound derivatives; and Boutwell, Brush, and Rusch[3] have shown that a single application of about this size induces papillomas in approximately 30 per cent of the mice of this stock if the hydrocarbon treatment is followed by repeated applications of the noncarcinogenic irritant, croton oil, for 200 days. Following 43 semiweekly applications of the carcinogen, about 67 per cent of these mice develop carcinomas at the site of treatment within 235 days.[3] Thirdly, the decreased levels of the protein-bound derivatives of 3,4-benzpyrene in the epidermis from mice exposed either to sunlight or to in-

[3] R. K. Boutwell, M. K. Brush, and H. P. Rusch, personal communication.

candescent lights are paralleled by the lower incidence of skin tumors in mice kept under similar conditions. Thus, Doniach and Mottram (3) found a marked inhibition of the formation of papillomas and carcinomas in mice treated topically with 3,4-benzpyrene when the mice were exposed to sunlight rather than being maintained in the dark, and Morton, Luce-Clausen, and Mahoney (13) obtained a similar decrease in tumor incidence when the mice were exposed to fluorescent lamps. Both the decreased levels of the 3,4-benzpyrene derivatives and the lower tumor incidence may be the result of photo-oxidation of a part of the carcinogen, so that the effective dose for the mice exposed to light was lower than for those maintained in the dark. If so, the binding of the hydrocarbon to the epidermal protein and the carcinogenic reaction are probably not mediated through surface oxidation of the hydrocarbon. Rather, it would appear that the hydrocarbon is taken into the cells and then metabolized to a derivative which unites with the proteins within the cells, and, possibly, is involved in the initiation of the carcinogenic process.

The means by which a reaction between derivatives of a carcinogen and cell protein might initiate a carcinogenic process can be visualized in the following manner. If the various proteins of the cell were attacked in a more or less random manner, some cells would undoubtedly lose so many or such vital proteins that the cells would die. However, among those cells which survived, a few might retain the capacity for growth but lose the capacity to respond to the growth controls of the body; these cells would be classed as tumor cells. Whether the proteins so affected were in nuclear genes, cytoplasmic genes (plasmagenes), or enzymes would seem to be of little importance so long as they were autosynthetic; in this way, the capacity for unlimited growth would be transmitted upon cell division. Furthermore, precancerous cells might arise by deletion or alteration of only a part of these critical proteins. If, in subsequent cell divisions, the rate of duplication of the remaining critical proteins did not keep pace with the rate of cell division, a few cells might eventually arise which completely lacked these proteins and might hence become the initial tumor cells. Such concepts as these can apply to any carcinogen and have already been discussed in more detail in connection with our studies on the protein-bound derivatives of the hepatic carcinogen 4-dimethylaminoazobenzene (8, 9, 14).

SUMMARY

1. Crude epidermal protein preparations from the skin of mice treated with benzene solutions of

3,4-benzpyrene contain fluorescent substances which appear to be derived from the carcinogen and combined through chemical bonds with the protein. The bound fluorescent derivatives were not removed from the epidermal protein preparations by exhaustive extraction with boiling solvents, and a high concentration of an acidic derivative and significant quantities of a neutral derivative were found on proteins which had been dissolved in dilute alkali and reprecipitated with trichloroacetic acid.

2. Both the neutral and acidic fractions could be extracted with benzene following alkaline hydrolysis in the presence of zinc dust, while only the acidic derivative was obtained when the zinc dust was omitted. Both derivatives were more polar than 3,4-benzpyrene, and the fluorescence of their benzene solutions was less sensitive to quenching by dissolved oxygen than the fluorescence of solutions of the carcinogen.

3. The protein-bound derivatives were found only in the epidermal fraction of 3,4-benzpyrene-treated skin. No protein-bound derivatives were found in the dermal portion of 3,4-benzpyrene-treated skin or in untreated skin or in kidney, heart, lung, liver, or intestine from benzene- or 3,4-benzpyrene-treated mice.

4. A single application of 300 μg. of the carcinogen gave rise to detectable levels of the bound derivatives within 3 hours and a maximum of 10 to 20 units of each derivative at 24 hours. Thereafter, the level dropped slowly; one-fourth of the maximum level was still present after 1 week, and none could be detected at 3 weeks. When 200 μg. of 3,4-benzpyrene was applied daily, a maximum level of about 40 units of each derivative was reached after four or five applications. If the mice were kept in direct sunlight or directly under incandescent lamps, the level of each derivative was reduced to one-tenth to one-third of that found in the skin of mice maintained in the dark or in diffuse light.

5. The protein-bound derivatives were not found following one or two applications of 3,4-benzpyrene to the skin of freshly killed mice.

6. Although preliminary, the data are consistent with the idea that the binding of 3,4-benzpyrene (or a derivative) with the epidermal protein might be involved in the genesis of skin tumors.

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The Anti-leukemic Action of Combinations of Certain Known Anti-leukemic Agents*

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The present study of the anti-leukemic action of combinations of certain rather diverse agents known to prolong survival time of mice with transplanted leukemia was prompted by the observation of an apparent anti-leukemic synergism between urethan and methyl-bis(2-chloroethyl)amine (9).

Urethan (3, 4, 5, 11), methyl-bis(2-chloroethyl)-amine (HN2) (3, 4, 9), benzene (3, 6), potassium arsenite (3, 6), colchicine (3), 4-aminopteroylglutamic acid (aminopterin) (2, 10), and x-radiation (6) have all been shown to alter the course of transplanted mouse leukemia. All the above-mentioned agents, with the exception of colchicine, have been used clinically with some degree of temporary palliative effect (7). In addition, 2,6-diaminopurine and 8-azaguanine have been recently shown to increase the life span of mice with certain strains of transmitted leukemia (1, 8, 10).

From a theoretical standpoint, it seemed worth-while to carry out certain trials of this nature to determine, if possible, which therapeutic agents were additive in their anti-leukemic activity. Such information might assist in determining the homogeneity of the mechanism of action of this most heterogeneous group of agents.

PROCEDURE AND RESULTS

As a first step, the acute LD_{50} of the nine therapeutic procedures was determined using CFW strain mice. The compounds were administered by the intraperitoneal route, and total body x-radiation was employed. The results of these acute toxicity determinations are presented in Table 1. These values are approximations obtained in most instances from three points on a log-probability plot.

An indication of the acute toxicity of certain dual combinations of these therapeutic agents was

then obtained by simultaneous administration of fractions of the LD_{50} of the various treatments (from different syringes). In the instances where x-ray was employed in combination with other

TABLE 1

ACUTE (SINGLE INJECTION) TOXICITY DATA ON KNOWN ANTI-LEUKEMIC AGENTS

Agent	Vehicle	Approximate LD_{50} (mg/kg)
Aminopterin	Saline	8.3
Benzene	Peanut oil	1,150
Colchicine	Saline	2.4
2,6-Diaminopurine	10 per cent gum acacia*	Ca. 250
8 Azaguanine	10 per cent gum acacia*	Between 100 and 1,000
HN2	Saline	3.2
KAsO₃	Saline	18.0
Urethan	Saline	1,800
X-radiation		600 roentgen

* Pulverized and suspended in 10 per cent gum acacia.
NOTE: Observation period was uniformly 10 days.

TABLE 2

MAXIMUM DOSE OF CERTAIN ANTI-LEUKEMIC AGENTS TOLERATED BY MICE ON SEVEN SUCCESSIVE DAILY INTRAPERITONEAL INJECTIONS

Agent	Vehicle	Maximum tolerated dose (mg/kg)
Aminopterin	Saline	0.23
Benzene	Peanut oil	250
Colchicine	Saline	0.63
HN2	Saline	0.75
KAsO₃	Saline	4.5
Urethan	Saline	700
X-ray*		150 r
2,6-Diaminopurine	Gum acacia	100
8-Azaguanine	Gum acacia	31

* Given twice within a 2-week interval with no deaths. This is not a maximum tolerated dose.

materials, administration of the compounds preceded the x-radiation by 30–60 minutes.

With knowledge of the maximum tolerated dose of the individual agents for seven successive daily injections (previously determined for anti-leukemic assays, Table 2), it was possible to select combination doses that, if not synergistic with regard to toxicity, might be used in anti-leukemic

TABLE 3

ANTI-LEUKEMIC SCREENING DATA ON CERTAIN COMBINATIONS OF KNOWN ANTI-LEUKEMIC AGENTS

Exp. no.	Treatment	Dosage (mg/kg)	Average survival (days)	Deaths, extremes in days	Per cent increase survival over untreated controls	R.C.I.*
1:	Controls		12.3	11–16		
	Aminopterin (Am)	0.23	20.4	16–28	+ 65.8	1.0
	Urethan	700	16.2	14–35	+ 31.7	0.48
	HN2	0.75	15.3	11–28	+ 24.4	0.37
	Am+urethan	0.12+225	19.3	16–23	+ 48.8	0.74
	Am+HN2	0.12+0.5	19.5	11–28	+ 58.5	0.89
	Urethan+HN2	450+0.38	19.0	14–28	+ 54.5	0.83
2:	Controls		12.7	11–17		
	Aminopterin (Am)	0.23	19.1	15–22	+ 50.4	1.0
	Colchicine	0.63	16.4	11–35	+ 29.1	0.58
	Am+colchicine	0.12+0.31	19.1	14–35	+ 50.4	1.0
	Am+benzene	0.12+125	16.5	14–23	+ 29.9	0.59
	Am+KAsO3	0.12+2.3	18.5	14–22	+ 45.7	0.91
	Am+x-ray†	0.12+150 r (twice)	17.5	14–28	+ 37.8	0.75
3:	Controls		11.0	9–13		
	Aminopterin (Am)	0.23	17.5	12–25	+ 59.1	1.0
	Am+colchicine	0.12+0.31	17.7	12–26	+ 60.9	1.0
4:	Controls		12.0	10–15		
	Aminopterin (Am)	0.23	19.1	17–25	+ 59.2	1.0
	HN2	0.75	17.3	12–30	+ 44.2	0.75
	Benzene	250	12.1	9–17	+ 0.8	0.01
	X-ray	150 r (twice)	12.9	12–15	+ 7.5	0.13
	KAsO3	4.5	13.6	11–18	+ 13.3	0.23
	Am+benzene	0.12+125	16.0	14–20	+ 33.3	0.56
	Am+KAsO3	0.12+2.3	16.4	13–26	+ 36.7	0.62
	Am+x-ray	0.12+150 r (twice)	17.9	14–23	+ 49.2	0.83
	Benzene+x-ray	250+75 r (twice)	13.3	9–20	+ 10.9	0.55
	KAsO3+x-ray	2.3+150 r (twice)	14.1	12–22	+ 17.5	0.30
	HN2+x-ray	0.5+150 r (twice)	15.9	13–18	+ 32.5	0.55
5:	Controls		10.0	9–11		
	Aminopterin (Am)	0.23	16.0	13–22	+ 60.0	1.0
	Colchicine	0.63	10.9	9–13	+ 9.0	0.15
	Am+colchicine	0.12+0.31	13.8	10‡–24	+ 38.0	0.63
6:	Controls		7.8	7–10		
	Aminopterin (Am)	0.23	15.6	10–23	+100.0	1.0
	Benzene	250	7.1	7–8	− 0.9	0.009
	X-ray	300 r (twice)	10.8	8–15	+ 38.5	0.39
	Urethan	700	10.1	7–15	+ 29.5	0.29
	HN2	0.75	11.0	9–15	+ 41.0	0.41
	Colchicine	0.63	8.7	7–9	+ 11.5	0.12
	KAsO3	4.5	8.2	7–10	+ 0.5	0.005
	2,6-Diaminopurine	100	8.8	7–10	+ 12.8	0.13
	Am+benzene	0.12+125	10.0	7–13	+ 28.2	0.28
	Am+x-ray	0.12+150 r (twice)	11.2	9–14	+ 43.6	0.44
	Am+urethan	0.12+225	12.2	11–14	+ 56.4	0.56
	Am+HN2	0.12+0.5	12.3	11–16	+ 57.7	0.58
	Am+colchicine	0.12+0.31	12.7	10–15	+ 62.8	0.63
	Am+KAsO3	0.12+2.3	11.9	9–14	+ 52.6	0.53
	Am+2,6-diaminopurine	0.12+50	12.1	8–14	+ 55.1	0.55
	Urethan+HN2	225+0.5	11.7	10–14	+ 50.0	0.50
7:	Controls		8.8	7–14		
	Aminopterin	0.23	16.8	14–20	+ 90.9	1.00
		0.18	15.7	14–19	+ 78.4	0.86
		0.12	13.9	10–17	+ 58.0	0.64
		0.06	9.8	8–13	+ 11.4	0.13
	Urethan	700	11.3	9–13	+ 28.4	0.31
	HN2	0.75	11.1	10–13	+ 26.1	0.29
	2,6-Diaminopurine	125	14.7	14–18	+ 67.0	0.74
	8-Azaguanine	2.0 (twice daily)	8.6	7–10	− 2.3	
	Urethan+HN2	350+0.38	12.7	11–22	+ 44.3	0.49
	2,6-Diaminopurine+8-azaguanine	50+2.0 (twice daily)	8.8	7–11	0.0	0.0
8:	Controls		8.1	7–9		
	A-methopterin	2.0	20.3	16–37	+150.6	1.00
	A-methopterin+8-azaguanine	2.0+10	17.8	12–28	+119.8	0.79

* R.C.I.=Per cent increase in survival of a candidate compound or combination/Per cent increase in survival of aminopterin (or A-methopterin).

† X-ray was in all instances administered on the second and the ninth days after inoculation with leukemic spleen brei.

‡ Colchicine given at 0.625 mg/kg by mistake +0.12 mg/kg of aminopterin on ninth day. This probably caused early deaths.

screening of combinations of agents. The selected combination doses were then checked for toxicity on groups of four mice each. The highest levels of the two agents causing no deaths on 10 alternate day administrations followed by a 10-day observation period were selected for anti-leukemic screening trials. It was not the purpose of the present effort to determine accurately the acute and cumulative toxicity of the combinations of agents studied. Rather, it was intended to gain information as to doses of combinations of important chemotherapeutic agents which could be used in screening against transmitted mouse leukemia. Briefly, none of the combinations appeared to be profoundly synergistic with regard to toxicity in this cumulative toxicity screening.

After having obtained the necessary toxicity data, a number of combinations of the above-mentioned agents were screened for anti-leukemic activity, by the use of a procedure that has proved generally satisfactory in several laboratories (3, 8, 9). The inbred mice employed were of the Akm strain (brother-sister inbred for genetic control). The leukemia transplanted in this study was the rather acute AK4 strain which kills untreated mice in about 7–12 days. In the usual assay, a large group of Akm mice received intraperitoneal inoculations of 0.1 cc. of leukemic spleen brei that contained about one million cells. Two days later, these animals were divided into an appropriate number of experimental groups of ten mice each. One group was retained as an untreated control; other groups were treated, starting on the second post-inoculation day, at the maximum tolerated level with the agent or combination of agents under study.

The results of these assays are reported in terms of the average per cent of increase in life span of treated over untreated groups of mice (Table 3).

DISCUSSION

As can be seen in Table 3, emphasis in this investigation has been centered around an attempt to increase the anti-leukemic action of aminopterin or A-methopterin in this rather acute strain of mouse leukemia. We have observed no indication of synergism (in Ak4 leukemia) between the following pairs of agents:

1. Aminopterin+urethan
2. Aminopterin+nitrogen mustard (HN2)
3. Aminopterin+colchicine
4. Aminopterin+benzene
5. Aminopterin+potassium arsenite
6. Aminopterin+x-radiation
7. Aminopterin+2,6-diaminopurine

8. A-methopterin+8-azaguanine
9. Benzene+x-radiation
10. Potassium arsenite+x-radiation
11. Nitrogen mustard (HN2)+x-radiation

In three experiments where the combination of urethan plus nitrogen mustard was employed, it appears that we have confirmed (using a different leukemic strain) the synergistic anti-leukemic effect between these two compounds, which was previously reported (9). However, in this rather acute leukemia (Ak4) the combination of urethan plus nitrogen mustard is not as effective as are the anti-folic acid type compounds.

The present data are not sufficient to allow for deduction with regard to additive effects of the various combination therapy, except perhaps to indicate that generally one-half the maximum tolerated dose of aminopterin plus one-half the maximum tolerated dose of the other agents studied was not as effective as the maximum tolerated dose of aminopterin alone, except perhaps in the case of aminopterin plus colchicine.

SUMMARY

A number of combinations of known anti-leukemic agents has been screened against mouse leukemia in search of synergistic activity. Only urethan plus nitrogen mustard exhibited evidence of synergistic activity, and this combination was less effective than aminopterin or A-methopterin for the rather acute Ak4 strain of leukemia used in these studies.

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Neoplasms in Rats Treated with Pituitary Growth Hormone

IV. Pituitary Gland*

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This paper is concerned with the changes occurring in the pituitary glands of rats receiving growth hormone over long periods. The previous papers in this series have dealt with the neoplasms occurring in the lungs, lymphatic tissues, adrenal glands, and reproductive organs of these rats (5–7).

A group of fifteen female rats of the Long and Evans strain, 237–239 days old, which had reached a plateau in weight, received intraperitoneal injections of pituitary growth hormone (4) daily, 6 days weekly, for a maximum period of 485 days. The initial daily dosage was 0.4 mg.; this was increased at intervals to a maximum of 3.0 mg. daily. The fifteen controls received comparable amounts of serum albumin.

The pituitary glands of eleven experimental and fifteen control rats were fixed in Zenker-formol and imbedded in nitrocellulose. Multiple sections, 4 μ thick, were taken from each of five different levels in the coronal plane. The sections were stained with a modified Mallory-Azan technic (1).

OBSERVATIONS

Controls

The pituitary glands of thirteen of the fifteen controls were macroscopically normal. In two of the control rats, the pituitaries were enlarged. The pituitary gland of one of these rats (B6508) weighed 33.7 mg.; the anterior lobe was cystic on the right and hemorrhagic on the left. The pituitary gland of the other rat (BH6262) weighed 177.6 mg.; there was extensive hemorrhage in the anterior lobe. The grossly normal pituitary glands varied in weight from 9.4 to 15.7 mg., with an average weight of 12.49 mg. ± 0.61.

The pituitary glands of seven of the fifteen controls were microscopically normal (Fig. 1). There were certain minor variations in the number and morphologic characteristics of the various types of cells in these normal glands. These variations, however, were attributable to the differences in the stages of the rats' estrous cycle at the time of autopsy and corresponded closely to those reported by Wolfe et al. (10) in their studies on the pituitary glands of old female rats of the Vanderbilt and Wistar strains.

In the anterior pituitaries of eight controls there were focal to large adenomatous areas composed of atypical cells which were quite unlike those present elsewhere in the surrounding parenchyma. The adenomatous areas were solitary in four rats and multiple in four. The morphologic characteristics of the various lesions are given in Table 1.

In three rats the lesions were focal and were composed of atypical chromophobic and acidophilic cells, which were usually larger than normal. Some of the cells were as much as 50 μ in diameter. These cells had sharply outlined cell membranes and abundant cytoplasm and were recognizable as modified chromophobes or acidophils. The rare basophils present in these lesions were normal. In the abnormal cells the negative image of the Golgi apparatus was frequently hypertrophied and quite complex. There was moderate variation in the granularity of the cytoplasm. The nuclei were vesicular and often considerably larger than normal. Lobulation of the nuclei was occasionally observed. Nucleoli were frequently greatly enlarged and occasionally multiple (Fig. 2). The parenchyma immediately adjacent to these foci was normal.

The larger adenomatous areas caused compression of the surrounding parenchyma. In some of the adenomata the capillaries were collapsed, giving the entire area an avascular appearance; in others the capillaries were greatly dilated. In one

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rat (GH6442) the vascular dilatation had progressed to the formation of a lacuna lined by endothelial cells (Fig. 3). In another rat there were small areas of old hemorrhage in the adenoma. These areas were characterized by collections of macrophages filled with hemosiderin.

Some of the larger adenomata were composed of modified chromophobes, some of modified acidophils, and still others of both chromophobes and acidophils. Several of these lesions were composed chiefly of hypertrophied acidophils of uniform size

abnormal, as stated above, one weighing 33.7 mg. (B6508) and the other weighing 177.6 mg. In the anterior pituitary of rat B6508 there were two adenomata composed of small chromophobes. One of the adenomata was hemorrhagic (Fig. 6), and the other was characterized by numerous cysts and small hemorrhagic areas (Fig. 7). In many of the hemorrhagic areas there were collections of macrophages filled with hemosiderin and other phagocytized particles. The neoplastic cells showed a tendency to be arranged in cords. The pituitary gland

TABLE 1

PITUITARY GLANDS OF CONTROLS INJECTED WITH ALBUMIN

ANIMAL NO.	AGE AT AUTOPSY (DAYS)	DAYS INJECTED	Weight (mg.)	PITUITARY GLAND			Alterations in basophils
				Size (μ)	Adenomata (no.)	Type	
B6530	615	378					
B6512	672	435					
B6400	722	484	10.7				
B6508	720	483	33.7	2,500*	2	Chromophobic	
W6523	720	483	13.0	400	2	Acidophilic	
GH6442	722	485	11.8	550	1	Acidophilic; vacuolated cells	
GH6554	722	485	15.6				
GH6357	722	484	9.4	350	3	Mixed†	
G6460	722	485	12.7	focal	1	Mixed†	
BH6262	722	483	177.6	5,500‡	1	Chromophobic	
BH6268	723	484	15.7	1,300	3	2 Chromophobic 1 Acidophilic	
B6291	722	484	13.3	focal	1	Mixed†	
BH6292	722	484	11.0				
B6492	722	485	10.6				
BH6294	723	485	13.7				

* One adenoma was hemorrhagic; the other contained multiple cysts.
† The "mixed" lesions were composed of atypical chromophobes and acidophils.
‡ This adenoma was hemorrhagic.

(Fig. 5). The nuclei of these cells were round and vesicular and contained a single prominent centrally placed nucleolus. Hypertrophy of the Golgi apparatus and mitoses were frequently seen. The cytoplasm of these cells showed all stages of degranulation, varying from moderate to complete. The cells with completely degranulated cytoplasm ("chromophobes") could be readily distinguished by their size and nuclear characteristics from the occasional normal chromophobes which were also present. These lesions closely resembled the adenomata observed in this laboratory following the administration of estrogens to rats (unpublished data) and hamsters (2).

In one rat (GH6442) there was an acidophilic adenoma which presented unique features (Fig. 4). The acidophils showed varying stages of degranulation and vacuolization of the cytoplasm. Nearly all cells had enlarged nuclei, with prominent and occasionally multiple nucleoli. A few tumor giant cells, measuring as much as 52 μ in diameter, were present.

In two rats the anterior pituitaries were grossly

of rat BH6262 weighed 177.6 mg. and was completely replaced by a hemorrhagic and cystic adenoma. This lesion was composed of moderately hypertrophied chromophobes of uniform size. The Golgi apparatus was occasionally hypertrophied. Occasional mitoses were present. The neoplastic cells were arranged in irregular cords or peritheliomatous structures (Fig. 8). There were large amounts of colloidal material and blood in between the groups of neoplastic cells.

The pars intermedia contained cysts of various sizes. The posterior lobes showed no abnormalities.

EXPERIMENTAL RATS

The pituitary glands of the rats injected with growth hormone varied in weight from 13.0 to 22.8 mg., with an average weight of 18.11 mg. ± 1.24. Although there were no grossly visible abnormalities of the pituitary glands, all the anterior pituitaries of the eleven experimental rats were abnormal microscopically (Table 2). Focal to moderately large adenomatous areas were found in the anterior pituitaries of all experimental rats. In the

areas of the anterior lobe not involved by adenomatous changes, the acidophils were decreased in number and in size; the decrease in size was associated with a loss of cytoplasmic granules. The chromophobes were increased in number. These findings were essentially similar to those previously reported from this laboratory (3). The basophils were normal in the pituitaries of eight experimental rats. In three rats (G6534, G6553, BH6313), there were advanced castration changes. In one of these (G6553), "signet-ring" forms comprised 33 per cent of all basophils (Fig. 11).

occasionally showed lobulation or budding. Nucleoli were not hypertrophied.

In three rats (G6271, B6269, B6266), the adenomata were composed almost entirely of degranulated acidophils and were morphologically identical with those found in the controls (Fig. 5). As stated above for the controls, the uniform size of the cells, hypertrophied Golgi, and nuclear characteristics gave these lesions a striking similarity to those resulting from estrogen administration.

The pars intermedia and posterior lobes were similar to those of the control pituitaries.

TABLE 2

PITUITARY GLANDS OF EXPERIMENTAL RATS INJECTED WITH GROWTH HORMONE

ANIMAL NO.	AGE AT AUTOPSY (DAYS)	DAYS INJECTED	Weight (mg.)	Size (μ)	PITUITARY GLAND Adenomata (no.)	Type	Alterations in basophils
G6534	587	350		focal	multiple*	Basophilic	"Castration changes"
B6458	615	378		focal	multiple	Acidophilic and Chromophobic	
BH6276	673	435		focal	1	Mixed†	
G6553	702	465		focal	multiple	Mixed†	"Castration changes"
BH6313	722	484	17.2	focal	3	Mixed†	"Castration changes"
W6324	721	483	15.8	560	2	Mixed†	
G6255	724	485	21.8	focal	multiple	Mixed†	
G6361	722	484	17.8	focal	1	Mixed†	
G6266	723	484	18.9	1,250	2	Acidophilic	
G6269	723	484	13.0	800	1	Acidophilic	
G6271	723	484	22.8	1,300	2	Acidophilic	

* Uniformly small lesions.
† The "mixed" lesions were composed of atypical chromophobes and acidophils.

Histologically, the adenomatous lesions in ten of the animals were essentially similar to the lesions of similar size occurring in the controls, except as described below. In one rat there were many small groups of atypical cells, as well as numerous isolated atypical cells. The individual cells of the adenomata in the experimental rats frequently were larger and more pleomorphic than those occurring in the controls. The nuclei were often larger, more bizarre, and contained irregular chromatin clumps. Nucleoli were very prominent (Figs. 9, 10, 12). The cytoplasm was abundant and showed varying degrees of degranulation. Multiple adenomata of the anterior lobe occurred more frequently in the experimental rats. No hemorrhagic areas were found in the experimental rats.

The pituitary gland of one rat (G6534), in addition to showing marked castration changes, contained numerous adenomata of uniformly small size. These were composed almost entirely of large, atypical basophils (Fig. 12). Some of the cells had a hyaline cytoplasm. Occasionally, there was moderate hypertrophy of the Golgi apparatus. The nuclei were large, frequently hyperchromatic, and

DISCUSSION

Neoplasms of organs other than the pituitary gland occurred much more frequently in the rats injected with growth hormone than in the controls (5–7). Because of the higher incidence of abnormalities of the anterior pituitary in the rats injected with growth hormone, the possibility of a correlation between this latter finding and the neoplasms of other organs was considered (Tables 3 and 4). The relationship of specific changes in the pituitary glands with neoplasms of other organs is discussed below.

The acidophils of the anterior pituitaries of all experimental rats were smaller, diminished in size, and degranulated; the chromophobes were increased in number. Thus, it seems obvious that there is no specific correlation between these changes and any given neoplastic lesion.

In the three experimental rats with typical "castration changes" of the anterior pituitary, there were atypical ovarian follicles. One of these rats also had a large adenocarcinoma of endometrial origin, and another had multiple fibroadenomata of the mammary gland. However, it

should be noted that comparable abnormalities of the reproductive organs were present in both control and experimental rats which had no abnormalities of the basophils.

Focal to large adenomatous lesions of the anterior pituitary occurred in eight of fifteen controls. This high incidence is in all probability associated with the advanced age of these animals. Wolfe *et al.* (10), Oberling *et al.* (8), and Saxton (9) have reported a high incidence of pituitary adenomata in old rats of other strains. The experimental rats injected with growth hormone showed an even higher incidence of pituitary adenomata. However, there was no demonstrable correlation between these adenomata and neoplasms of any given type occurring in the other organs.

Thus, there is no apparent correlation between any specific abnormality of the pituitary gland and neoplasms of any given type in the other organs. Further studies will be necessary to clarify the mechanism of action of growth hormone in producing the lesions described in this and the preceding papers.

SUMMARY AND CONCLUSIONS

1. The pituitary glands of eleven rats injected for long periods with pituitary growth hormone and of fifteen controls injected with serum albumin were studied.

2. The acidophils of the anterior lobe of rats injected with growth hormone were smaller, less numerous, and contained fewer granules than those of the controls.

TABLE 3

CONTROL RATS. PITUITARY LESIONS AND NEOPLASTIC CHANGES OF OTHER ORGANS

| ANIMAL NO.	PITUITARY GLAND Adenomata	Basophilic changes	LUNGS AND LYMPHATIC TISSUE	ADRENAL MEDULLA	OVARY	UTERUS	MAMMARY GLAND
B6530			Lymphosarcoma of ileocecal nodes				
B6512						Adenocarcinoma of endometrium	
B6400							
B6508	+						Fibroadenoma
W6523	+		Pulmonary adenomatosis				Fibroadenoma
GH6442	+						
GH6554							
GH6357	+						
G6460	+				Cystadenoma		
BH6262	+						
BH6268	+						Fibroadenoma
B6291	+						
BH6292							
B6492							
BH6294							

TABLE 4

EXPERIMENTAL RATS. PITUITARY LESIONS AND NEOPLASTIC CHANGES OF OTHER ORGANS

ANIMAL NO.	PITUITARY GLAND* Adenomata	Basophilic changes	LUNGS AND LYMPHATIC TISSUE	ADRENAL MEDULLA	OVARY	UTERUS	MAMMARY GLAND
G6534	+	+†			Atypical follicles	Adenoma of endometrium	
B6458	+		Lymphosarcoma		Cystadenomata		
BH6276	+			Pheochromocytoma			Fibroadenomata
G6553	+	+†	Lymphosarcoma		Atypical follicles		
BH6313	+	+†	Lymphosarcoma	Pheochromocytoma	Atypical follicles		Fibroadenomata
W6324	+		Lymphosarcoma	Pheochromocytoma	Multiple tumors		Fibroadenomata
G6255	+						Fibroadenomata
G6361	+			Pheochromocytoma			
B6266	+						
G6269	+			Pheochromocytoma			Fibroadenomata
G6271	+		Lymphosarcoma	Pheochromocytoma	Atypical follicles		

* The acidophils in non-adenomatous areas of the anterior pituitary of all experimental rats were decreased in size and number, and the cytoplasm contained fewer granules.

† Typical late castration changes.
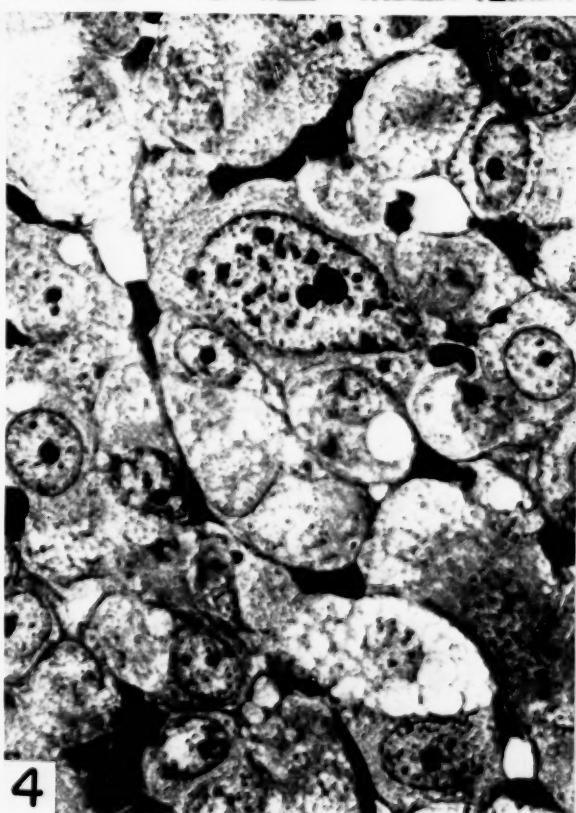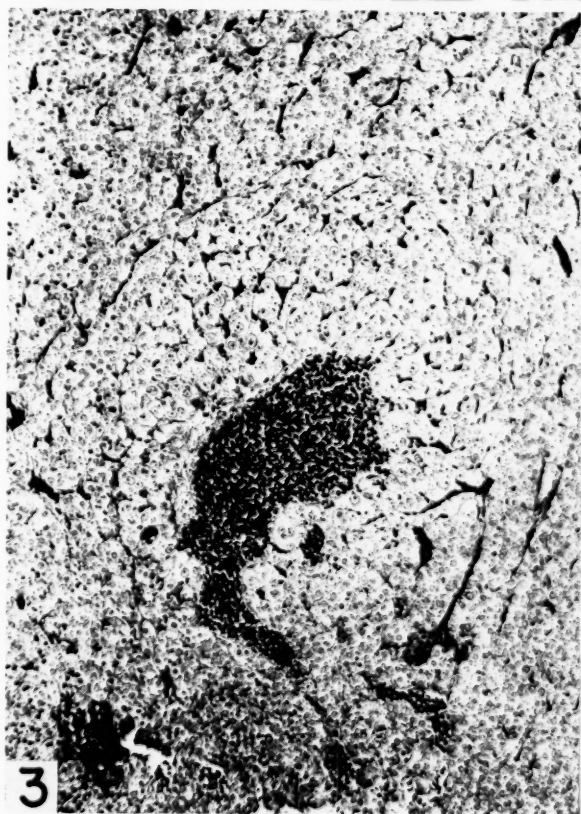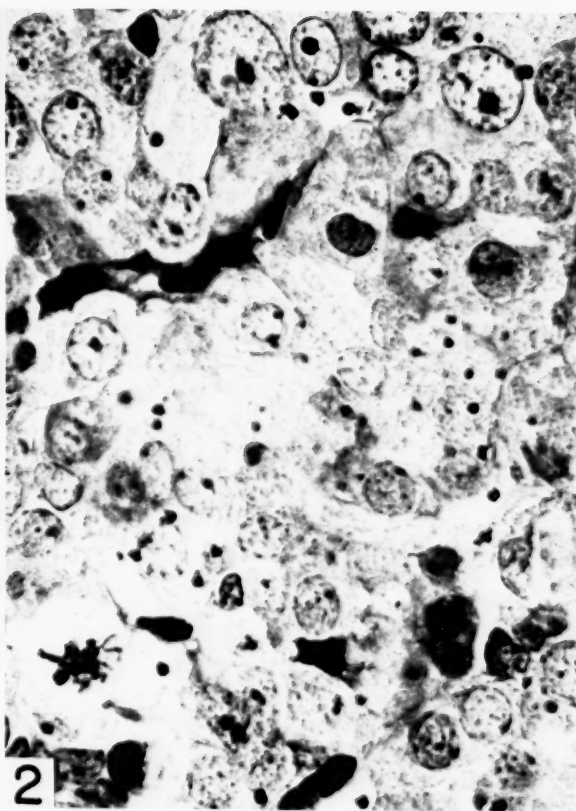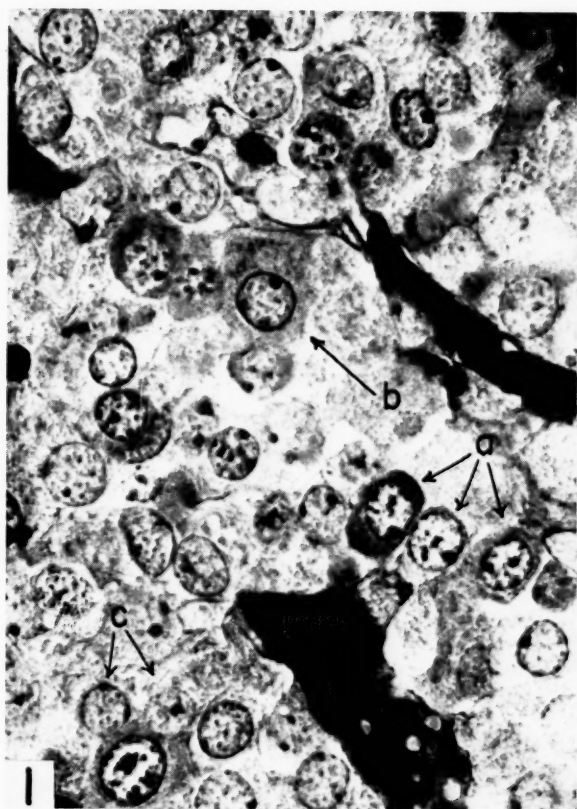
FIGS. 1–8.—Controls; anterior pituitary; Mallory-Azan.

FIG. 1.—BH6294; normal. Note distribution and size of cells: a = acidophils; b = basophils; c = chromophobes. Mag. ×1,000.

FIG. 2.—G 6460; focal adenoma. Most of the cells are degranulated acidophils or chromophobes which are larger than normal. The nuclei vary greatly in size; nucleoli are large. There is a mitotic figure at the lower left. Mag. ×1,000.

FIG. 3.—GH 6443; small adenoma. The central area of the poorly circumscribed adenoma shows a lacuna filled with blood. Mag. ×110.

FIG. 4.—GH 6442. Higher magnification of the lesion shown in Figure 3 showing vacuolization of cytoplasm, hypertrophy, and variation of nuclei. Nearly all the cells also contain acidophilic granules. Mag. ×1,000.

FIG. 5.—GH 6357. Adenoma. This lesion is composed of both acidophils and chromophobes. The cells are moderately hypertrophied but quite uniform in size. The nuclei are round and vesicular; nucleoli are centrally placed and prominent. The acidophils are in various stages of degranulation. The chromophobes probably represent completely degranulated acidophils. Mag. ×450.

FIG. 6.—B 6508; hemorrhagic chromophobe adenoma. Area showing hemosiderin-filled macrophages and erythrocytes. Mag. ×450.

FIG. 7.—B 6508; cystic chromophobe adenoma. There are many cysts filled with colloid. Note numerous small darkly staining hemorrhagic areas. In many areas the cells are arranged in cords. Mag. ×100.

FIG. 8.—BH 6262; hemorrhagic chromophobe adenoma. This tumor shows a peritheliomatous pattern. Neoplastic cells of uniform appearance are clustered around small vessels. Note the extravasated red blood cells in between the cords of neoplastic cells.
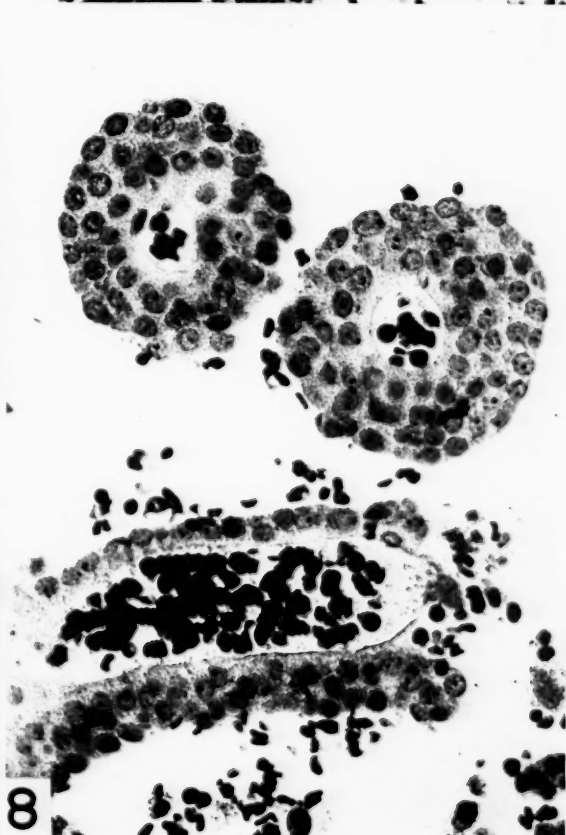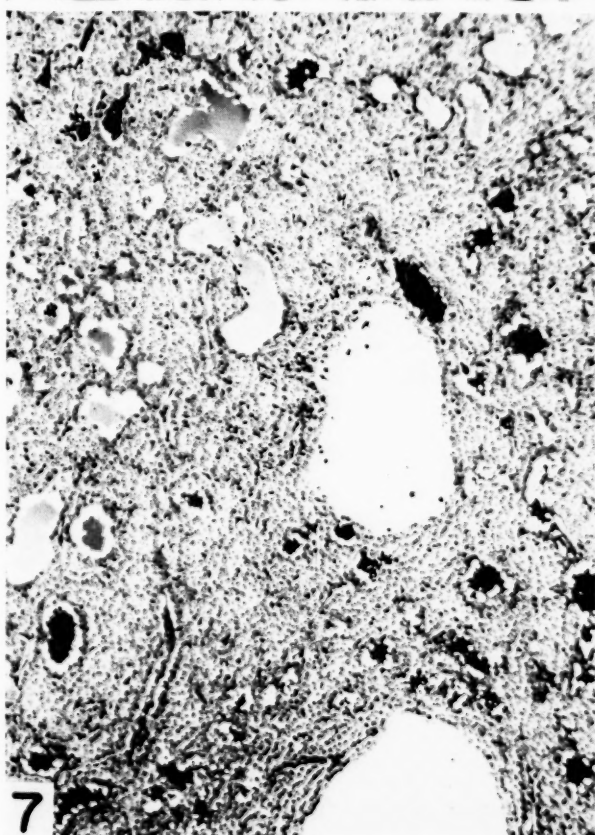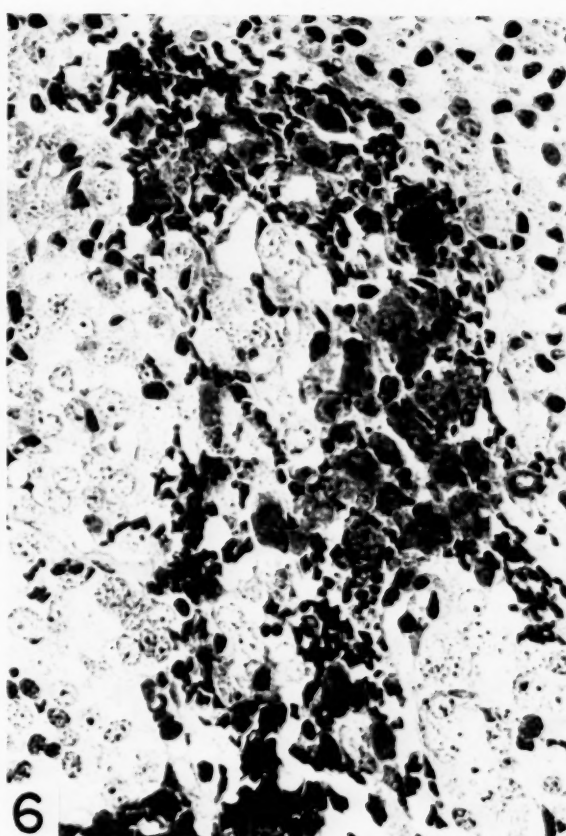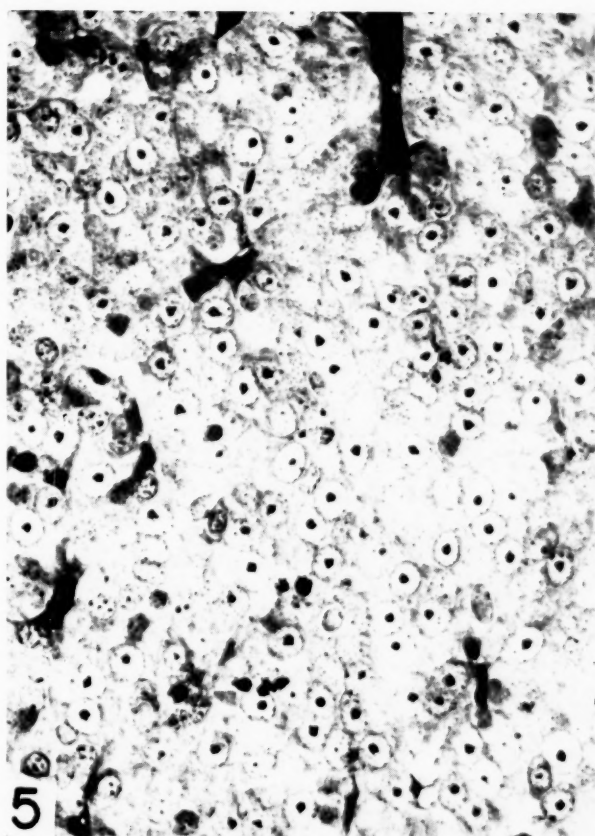
FIGS. 9–12.—Experimental rats; anterior pituitary; Mallory-Azan.

FIGS. 9 and 10.—BH 6313. Focal "mixed" adenomata. Tumor giant cells. (Compare with Figs. 1, 2, and 4.) Mag. ×1,000.

FIG. 11.—G 6553. "Castration changes." Note (a) normal basophils and (b) "signet-ring" forms.

FIG. 12.—G 6534. Focal basophilic adenoma. This lesion consists of large basophils with abundant cytoplasm and hypertrophied nuclei. The nucleoli are not prominent. There are many bizarre, hyperchromatic nuclei. Several "signet-ring" basophils are seen along the lower edge of the field.
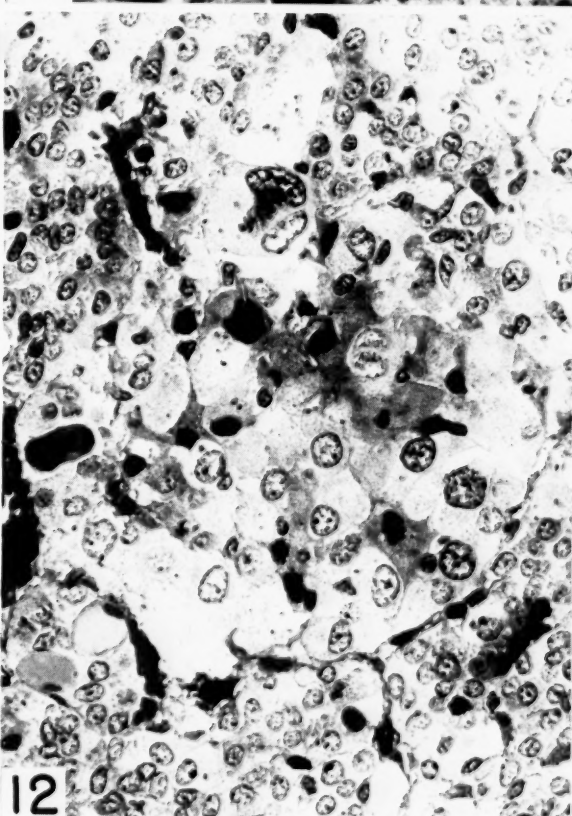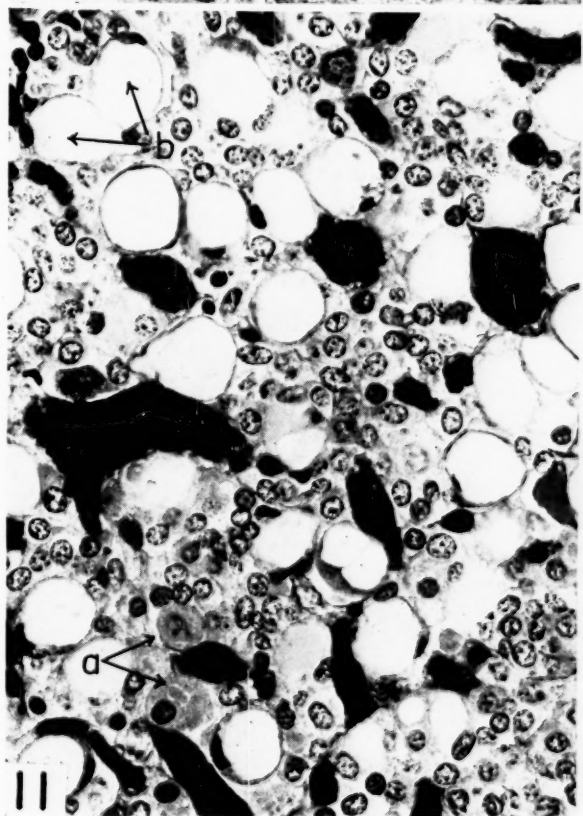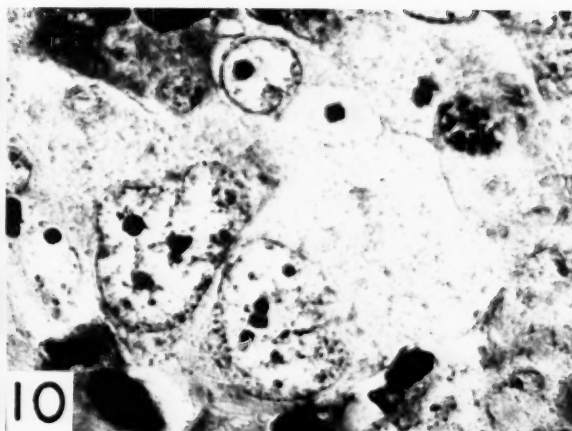
3. In three of eleven experimental rats, the basophils were increased in number and were similar to those occurring in castrate rats. In one of these rats there were numerous small basophilic adenomas. No similar changes occurred in the controls.

4. The adenomatous lesions of the anterior pituitaries were more numerous and were present in all of the eleven experimental rats which were studied. Similar lesions occurred in only eight of fifteen controls.

5. There was no correlation between specific pituitary lesions and the neoplasms of any given type occurring in the other organs.

6. No significant changes were noted in the pars intermedia or posterior lobe.

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Phosphorylated Intermediates in Glycolysis of Analogous Mouse Mammary Tumors*

I. Mouse Mammary Tumors of the dba and C3H Strains

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INTRODUCTION

In previous communications by one of the authors (5–9), it was shown that mammary tumors, histologically diagnosed as adenocarcinomas, although morphologically almost identical, differed in their rate of growth, over-all metabolic activity, and radiosensitivity. The mammary tumors investigated are the dbrB tumor, autogenous to the dba strain of mice, and the C3H tumor autogenous to the C3H strain of mice. For the sake of brevity these two tumors will be referred to as the dbrB and C3H tumors, respectively.

As was reported, the rate of growth was judged by the latent period, i.e., the time elapsing between the implantation of a tumor graft and the detection of tumor growth. This latent period was about 4–6 days for the dbrB tumor and about 14–18 days for the C3H tumor.

Furthermore, the metabolic rates, as determined by the oxygen intake and aerobic glycolysis *in vitro* (Warburg manometric technic), were as follows: for the dbrB tumor, the rate of oxygen intake (Q_{O_2}) averaged 5.9 and the aerobic glycolysis ($Q_{Lac}^{O_2}$) averaged 24.3. For the C3H tumor, the rate of oxygen intake (Q_{O_2}) averaged 3.6 and the aerobic glycolysis ($Q_{Lac}^{O_2}$) averaged 8.5, determinations being done in 100 per cent oxygen. Thus, as measured by $Q_{Lac}^{O_2}$, the dbrB tumor was found to have a rate of growth and rate of metabolic activity about 3 times that of the C3H tumor.

Attempts are being made to compare the properties of these tumors by more detailed studies, so as to account for the differences in their biological and physiological behavior. One of these studies will be reported here.

As previously mentioned, the mammary tumors, dbrB and C3H, exhibited a significant difference in their respiratory and over-all glycolytic activities *in vitro*. It was deemed of interest to investigate the pathways of the whole glycolytic cycle of the actively growing tumors *in vivo*. This was accomplished by determining the various components encountered in this cycle by the use of the freezing technic and modern enzymatic and chemical methods. To the knowledge of the authors, no such data on these particular tumors are available in the literature.

EXPERIMENTAL

Young inbred mice of the dba and C3H strains received, by trocar, grafts of a small tumor particle, autogenous to the strain, which was placed subcutaneously between the groin and subaxillary region. When tumors of a measurable size appeared, the mice were anesthetized by an intraperitoneal injection of nembutal so as to immobilize the animal. To exclude necrotic portions usually present in the center of the tumor, only surface portions of the actively growing tumors were dissected and immediately placed in either liquid air or in an ether and dry ice mixture. The frozen tumor tissue was pulverized in a specially constructed brass chamber by a heavy piston and was immediately placed in a small ice-cold beaker containing the necessary amount of 10 per cent trichloroacetic acid. Since the weight of the beaker plus the trichloroacetic acid had been determined previously, the amount of the tumor sample taken for analysis was thereby calculated by the difference in the two weights. About 1–2 gm. of tumor tissue, taken from surface portions of 2–3 tumors,

proved to be adequate for the determinations of the components involved in the Embden-Meyerhof scheme of the glycolytic cycle.

The known amount of pulverized tumor suspended in trichloroacetic acid was immediately transferred quantitatively into a Pyrex glass homogenizer of the Potter-Elvehjem (16) type in order to obtain as complete an extract of the tissue as possible. Strict precautions were taken to keep the tumor material for analysis ice-cold during all procedures requiring low temperature. When the tumor tissue could not be analyzed immediately, it was stored in a deep freezer at about $-20°$ C.

The compounds determined included total acid-soluble phosphorus, inorganic orthophosphate, lactic acid, glycogen, glucose-1-phosphate, fructose-6-phosphate, fructose-1,6-diphosphate, phosphoglyceric acid, coenzyme I, adenylic acid, adenosine diphosphate, adenosine triphosphate, and phosphocreatine. The last two compounds are of special interest, since they represent the chief storehouse of immediately utilizable energy for tissues.

The methods for the determination of the individual components are essentially those described by LePage in the monograph "Manometric Techniques and Related Methods for the Study of Tissue Metabolism" (18) and used by him in his studies on phosphorylated intermediates in tumor glycolysis (12, 13). Fractionation procedure B was used. A slight modification in the determination of phosphopyruvic acid was introduced by determining the phosphorus without removing the excess iodine from solution. Adenylic acid, adenosine diphosphate, adenosine triphosphate, and CoI were assayed enzymatically by the method described by Albaum and Lipshitz (1). This method involves converting the higher nucleotides to adenylic acid by a combined hexokinase, myokinase, and nucleotide pyrophosphatase system and assaying the adenylic acid formed by enzymatic conversion to inosinic acid with a muscle deaminase preparation. In later determinations of CoI, the method of Racker (17) was used. In this procedure, the CoI is reduced by alcohol dehydrogenase and its concentration determined from the absorption at 340 mµ.

Adenylic deaminase (Schmidt's deaminase) was prepared according to the method of Kalckar (11); myokinase, according to that of Colowick and Kalckar (3). Hexokinase was prepared in partially pure form, according to the method of Berger, Slein, Colowick, and Cori (2) through step 3 (fractionation with ethanol at 0° C.) of the isolation procedure. The nucleotide pyrophospha-

tase was a gift from Dr. Kornberg[1] of the National Institutes of Health, and the alcohol dehydrogenase was received from Dr. Racker[1] of the New York University College of Medicine. The enzymes were kept in a deep freezer and were found to be stable over long periods of time.

RESULTS

The mean values of components considered in phosphorylative glycolysis obtained from 27 analyses carried out on dbrB tumors and from 22 analyses carried out on C3H tumors, along with the standard errors for each mean, are recorded in Table 1. The values of each component represent mg. in 100 gm. wet weight of tumor tissue.

TABLE 1

MEAN VALUES OF PHOSPHORYLATED INTERMEDIATES
OF C3H AND dbrB TUMORS

	C3H			dbrB	
	MG/100 GM WET TISSUE				
	N	Mean S.E.M.		N	Mean S.E.M.
Total phosphorus	22	55.8±3.29		27	52.2±1.34
Inorganic P	22	28.2±2.78		25	15.9±1.12
Phosphocreatine	17	31.6±4.50		17	27.6±3.65
Lactic acid	20	98.8±4.65		15	100.7±4.51
Phosphoglyceric acid	21	22.9±2.43		26	25.2±1.49
Glycogen	15	14.1±1.66		11	18.8±2.27
Hexose diphosphate	22	9.3±0.90		25	8.1±1.00
Fructose-6-P	22	4.9±0.95		22	3.7±0.31
Glucose-1-P	20	6.2±0.57		17	10.8±1.68
Adenylic acid	19	10.6±1.77		24	13.5±1.16
Adenosine diphosphate	13	24.2±4.80		18	21.7±4.91
Adenosine triphosphate	12	18.0±2.73		18	43.4±4.30
Coenzyme I	14	15.3±3.02		25	10.4±1.10

It should be mentioned that there is a difference in the water content of these tumors. As previously reported (8), the water content of the dbrB tumor averaged 85.5 per cent and that of the C3H tumor averaged 81.5 per cent. The difference in the water content of the tumors is in accord with the difference in their rate of growth, for it is known that faster proliferating tissues possess a greater water content than slower proliferating ones.

To determine whether the difference between the means of the values included in the table were statistically significant, diff/S.E. values were calculated using the formula for standard error for a difference: $S.E._{diff.} = \sqrt{S.E._1^2 + S.E._2^2}$. Ratios for diff/S.E. of greater than 2 were considered significant. An analysis of the data recorded in the table revealed the following: the over-all pattern of components of both tumors resembles that of

normal differentiated tissues according to the Embden-Meyerhof phosphorylative glycolysis system, with the exception of the lactic acid values, which are relatively higher than those of normal differentiated tissues. This may be explained by the high rate of glycolysis of rapidly proliferating tissues.

Significant differences between the mean values for the two mammary adenocarcinomas analyzed can be noted for inorganic phosphorus, glucose-1-phosphate, and adenosine triphosphate. The difference in glycogen is almost statistically significant, diff/S.E. resulting in 1.94. There is no significant difference in the mean values of the lactic acid content of the two tumors. This is not in accord with the results obtained from the experiments carried out *in vitro* and reported in a previous publication (8), showing that the dbrB tumor produced about 3 times as much lactic acid as the C3H tumor.

DISCUSSION

In the study of the levels of the phosphorylated intermediates in glycolysis of the analogous mouse mammary tumors, dbrB and C3H, it was found that a significant difference exists between the mean values of inorganic phosphorus, glucose-1-phosphate, and adenosine triphosphate. As mentioned above, the difference in glycogen was almost statistically significant, diff/S.E. resulting in 1.94, particularly if the difference in water content between the C3H tumor (81.5 per cent) and the dbrB tumor (85.5 per cent) is taken into account.

The higher glucose-1-phosphate level, the critical ratio being $+2.8$, in the dbrB tumor suggests a more rapid rate of glycogen breakdown in this tumor. Further, it was noted that the dbrB tumor, as compared to the C3H tumor, had a relatively lower level of inorganic phosphorus, the critical ratio being about -3, and a relatively higher level of adenosine triphosphate, the critical ratio being about $+5$. These findings strongly suggest that a higher energy level is available for vital function of the dbrB tumor. The observations made in this study are in accord with those previously reported (5–9), showing that, although these mammary tumors are morphologically similar, the dbrB grows more rapidly and has a higher rate of oxygen uptake than the C3H.

It is of interest to note in the table the almost similar mean values of lactic acid, which is 98.8 mg/100 gm $\pm$ 4.65 S.E.M. for the C3H tumor and 100.7 mg/100 gm $\pm$ 4.51 S.E.M for the dbrB tumor. This relatively small difference in the amount of lactic acid present in the tumors *in situ* is not in agreement with *in vitro* determinations. As mentioned in the introduction, the dbrB tumor produces about 3 times as much lactic acid *in vitro* as the C3H tumor (8). How can this discrepancy be accounted for? The following explanation may be offered:

In the dbrB tumor, with its inherent greater rate of growth and richer vascularity, the removal of metabolites takes place at a faster rate. Therefore, the lactic acid produced diffuses into the blood stream at a faster rate than that of the C3H tumor. Reference is here made to the work of Cori and Cori (4).

Furthermore, it has been shown that tumors are rich in buffers and that the lactic acid is immediately neutralized to potassium lactate. The above accounts for the fact that the pH of tumor tissue is not lowered to the extent expected, owing to the high glycolysis—even after the glucose level is raised significantly by administering a high carbohydrate diet (10).

It follows, therefore, that a natural physiological mechanism, regulating the acid-base system and preserving the status quo in large part, exists in tumors. In fact, should it be otherwise, the tumor would be destroyed by its own glycolytic hyperactivity, and the whole organism would suffer from a pronounced hyperacidity. This is not the case. The relatively small increase in lactic acid in the blood of tumor-bearing individuals, animal and human, occurs only when the tumor is in a far advanced stage of growth and disintegration simultaneously sets in.

That the levels of the phosphorylated intermediates occurring in glycolysis of tumors are qualitatively and quantitatively similar to those occurring in normal differentiated tissues may be explained on the same basis—i.e., on the preservation of the acid-base balance, to a great extent, in tumors *in vivo*, permitting the whole glycolytic cycle to proceed as in normal differentiated tissues.

SUMMARY

Data are presented on the levels of intermediates in the glycolytic cycle for two mouse mammary adenocarcinomas, one of which is normally rapidly growing (dbrB) and the other relatively slowly growing (C3H). The pattern of compounds present corresponds to that found in normal differentiated tissues, except that the lactic acid level is considerably higher in these tumors.

The more rapidly growing mammary tumor (dbrB) shows significantly higher levels of glucose-1-phosphate, adenosine triphosphate, and possibly glycogen, and a lower level of inorganic phosphorus than those of the C3H mammary tumor. These findings are in agreement with other studies

carried out with this tumor and strongly suggest that a higher level of energy is available for vital function in the dbrB tumor.

The significance of the *in vitro* and *in vivo* differences in the lactic acid production of the two mammary tumors is discussed.

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The Effects of Injections of Lyophilized Normal and Neoplastic Mouse Tissues on the Growth of Tumor Homoiotransplants in Mice*†

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The technic of transplantation has provided the experimenter in the field of cancer research with a tool for studying the processes characterizing neoplastic growth and for the controlled testing of procedures affecting the course of growth. The biological prerequisites for successful growth and survival of grafts appear to be basically the same for both tumors and normal tissue. In brief, the requirements are that both implant and host have compatible biochemical makeups, so that the graft does not evoke compensatory reactions on the part of the host which will act to destroy the "foreign" tissue. This precondition is best realized for autotransplants, or for those circumstances in which the donor and host are monozygotic twins or come from the same inbred strains, thus with identical or nearly identical hereditary makeups. (There are apparent exceptions to these generalizations in the case of the so-called nonspecific tumors, although even these grow better in some inbred strains than in others.)

However, following certain treatments of the host prior to grafting, *tumor* implants will survive in animals in which they would normally fail to grow. One experimental technic is the injection of tumor tissue, prior to grafting, which has been treated in different ways: storage in the frozen state, in paraffin or under glycerin at freezing temperatures over a period of time (1–3), or lyophilization (8, 9). Casey and his collaborators (1–3) found a significant increase in both the growth rate and the number of positive "takes"

for the Brown-Pearce tumor in rabbits and the mouse mammary carcinoma E 0771 in the C57 black-6 line of mice. Snell, Cloudman, *et al.* (8, 9), using a variety of mouse tumors and inbred lines of mice, obtained marked increases in both the rate of tumor growth and the number of surviving grafts. For certain combinations of tumor and inbred lines of mice, there was an apparent inhibition rather than enhancement of growth of the implant. The literature has been reviewed extensively (8).

Additional experiments by Kaliss and Snell (7) have shown that pre-injections of lyophilized *normal* tissues will enhance the growth of tumor homoiotransplants in unrelated strains of mice in which they would normally regress. The present report deals with the effect of injections of lyophilized normal and neoplastic tissues on the subsequent growth of tumor homoiotransplants.

MATERIALS AND METHODS

Two sets of experiments are to be reported. In the first, the inbred strains of mice were: donor strain, A (Lilly); host strains, C57 black-6 and C57 brown-cd. The latter two are derived from a single strain, but they are separated sufficiently far back in their breeding history so that transplants of a tumor indigenous to one strain will regress in 100 per cent of the hosts of the other. Both are unrelated to the A (Lilly) strain. The test tumor used was tumor 15091a, which is an anaplastic carcinoma (predominantly spindle-celled) indigenous to the inbred A strain of mice. This tumor, which was found in a strain A female at the Jackson Laboratory in 1928, was originally diagnosed as a mammary gland adenocarcinoma and later as a spindle-cell carcinoma. Grafts grow rapidly and kill 100 per cent of strain A mice of both sexes within 3–5 weeks after subcutaneous implantation. Occasionally, animals survive as long as 8 weeks. The tumor will also grow in a limited number of animals of several other inbred strains of mice.

In the second set of experiments, the donor and

host strains were C57 black-6 and B alb C (derived from the Bagg albino strain), respectively. The test tumor was the mammary gland adenocarcinoma E 0771, which was found in a C57 black-6 female at the Jackson Laboratory in 1940. It is a rapidly growing tumor which kills 100 per cent of C57 black-6 hosts in about 3 weeks after implantation. It will survive in a small percentage of the animals of several unrelated strains but regresses in 100 per cent of B alb C animals.

For all experiments, the animals were about equally divided by sex and ranged in age from 10 weeks to 4 months at the start. All females were virgins. The animals were housed with not more than five or six to a box. They were given Purina Fox Chow and water *ad libitum*. No infective history was followed, but all animals appeared vigorous and healthy at the start of the experiments.

ysis occurs in this mixture. Washing of the red blood cells was accomplished by successively spinning them down in the centrifuge and resuspending them in two changes of the sodium citrate–sodium chloride mixture and three changes of 0.85 per cent sodium chloride. After the last sodium chloride wash, the packed red cells, with the supernatant fluid drawn off, were shell-frozen in 50-ml. Erlenmeyer flasks and lyophilized.

For purposes of injection, the powdered tissues were put into aqueous suspension in glass homogenizers. In earlier experiments, 0.85 per cent sodium chloride was the suspending vehicle; in later experiments, double-distilled water was used. The vehicle used appeared to make no difference in the results, and double-distilled water was later used throughout as a convenience. All injections were given intraperitoneally in 0.5-ml. amounts. Controls were

TABLE 1

PROTOCOL OF INJECTIONS OF LYOPHILIZED TISSUES*

Exp. no.	Lyophilized substance injected	Injection intervals	No. of inject.	Amt. per injection (mg.)	Total amt. inj. per mouse (mg.)
1	Strain A liver, kidney, spleen, tumor 15091a	Weekly	4	10	40
2	Strain A liver, kidney, red blood cells, tumor 15091a	Semi-weekly	10	5	50
3	Strain A liver, kidney, tumor 15091a	"	10	5	50
	Red blood cells	"	10	10	100
4	Strain C57 black-6 liver, kidney, spleen, tumor E 0771	"	10	5	50

* Live tumor was inoculated subcutaneously 1 week after the last injection. In experiments 1, 2, and 3, the inoculum was strain A tumor 15091a; in experiment 4 it was strain C57 black-6 tumor E 0771.

The lyophilized tissues were liver, kidney, spleen, washed red blood cells, and the test tumors. The normal tissues were obtained from mice ranging in age from $2\frac{1}{2}$–4 months and about equally divided in sex. These animals were perfused with 0.85 per cent saline through the hepatic portal vein, while under nembutal anesthesia. The blood was let from a severed jugular vein. The animals which supplied the tumor tissue were not perfused. The tissues were then excised, cut into small pieces with scissors, and frozen on stainless steel screens in a CO_2-ice chest. Freeze-drying was carried out by the "cryochem" process (4). The dried tissues were powdered in mortars, sealed under vacuum in ampules, and stored under refrigeration. All operations were carried out under clean, though not absolutely sterile, conditions.

The washed red blood cells were prepared as follows: Blood was obtained by incision of the lateral tail vein; about 1 volume of blood was diluted with 4 volumes of a mixture consisting of equal parts of 2 per cent sodium citrate and 0.85 per cent sodium chloride. Very little or no hemol-

either uninjected or injected with 0.85 per cent saline. The injection protocols are given in Table 1.

One week after the last injection, single subcutaneous implants of bits of live tumor were made by trocar in the right suprascapular region. To establish the potency of the particular batch of live tumor used in each experiment, transplants were also made to mice of the indigenous inbred strain. The subsequent course of growth of the grafts was followed by palpation at semi-weekly intervals for the first 3 weeks after inoculation, and weekly thereafter. Two-dimensional outline sketches, approximating the size and shape of the grafts as they felt to the fingers, were made at each observation. The course of growth of the grafts was followed until the time the animals either died with a positive tumor growth or were completely negative for a consecutive period of about 2 months. The latter time interval was chosen after it was found in several instances that animals that appeared to be negative for a 1-month period suddenly began to show active tumor growth, which eventually killed them.

RESULTS

Experiments with tumor 15091a.—The combined data for three experiments (experiments 1, 2, and 3 of Table 1) with the strain A tumor are presented in Table 2. This table shows the materials injected, the total amount injected per mouse, the number of animals dying with tumors (given in numerators), the total number of animals in each group (given in denominators), and the per cent dying.

It is evident that positive "takes" of the grafts follow the injection of lyophilized normal and neo-

mice. Most of the mice died between the third and fifth weeks after inoculation. Four mice lived as long as 4 months. In two of these, the implants grew very slowly. In one mouse the graft, after initial growth, regressed to about pea size by the seventh week after inoculation and then started growing again, the animal dying by the sixteenth week after inoculation. In the fourth mouse, there was complete regression, as far as could be determined by palpation, 3 weeks after inoculation, and the animal remained apparently negative to the seventh week. At this time a growth could again

TABLE 2

THE EFFECT OF PRIOR INJECTIONS OF LYOPHILIZED TISSUES FROM STRAIN A (LILLY) MICE ON HOMOIO-
TRANSPLANTS OF THE STRAIN A TUMOR 15091a IN C57 BLACK-6 AND C57 BROWN-CD MICE*

LYOPHILIZED SUBSTANCE INJECTED	TOTAL AMT. PER MOUSE	HOSTS DYING WITH TUMORS*†					
		C57 black-6		C57 brown-cd		Total dying	
		No.	Per cent	No.	Per cent	No.	Per cent
Normal liver	40 or 50 mg.	8/31	26	2/10	20	10/41	24
Normal kidney	40 or 50 mg.	13/30	43	6/10	60	19/40	48
Normal spleen	40 or 50 mg.	9/14	64	9/10	90	18/24	75
Red blood cells	50 or 100 mg.	0/24	0			0/24	0
Tumor 15091a	40 or 50 mg.	28/35	80	5/9	56	33/44	75
Controls	Nothing, or 5 ml. 0.85 per cent saline	0/33	0	0/10	0	0/43	0

* Numbers in the numerators are the number dying with tumors; numbers in the denominators are the total animals in each experimental group. The data are combined for three experiments.

† Tumor 15091a grows progressively in 100 per cent of strain A mice. In the above experiments, all of 26 control mice of the A (Lilly) strain died with tumors.

plastic tissues, with the exception of lyophilized red blood cells. The reason for the latter exception is not known. (Subsequent experiments by one of us [N. K.] show that prior injections of lyophilized normal serum and plasma from strain A mice are followed by "takes" of the tumor in a significant number of C57 black-6 mice.)

From Table 2 it is seen that lyophilized tumor and spleen evoke about the same degree of response in the hosts receiving homoiotransplants of the tumor, followed in order of effect by kidney and liver. Data extracted from other experiments than those reported in Table 2 show that the effect of lyophilized tumor is equal in both C57 black-6 and C57 brown-cd hosts. These data are presented in Table 3.

The course of development of the tumor transplants was marked by an initial growth of the grafts in almost all animals, experimental and controls. In the controls and in mice receiving lyophilized red blood cells, these remained small and about 2 weeks after inoculation underwent rapid and complete regression. In the experimental groups, positive growth continued at a fast rate in those animals in which there were successful takes. Erosion of the skin and of the center of the underlying tumor mass occurred in many of these

TABLE 3

THE EFFECT OF INJECTIONS OF LYOPHILIZED STRAIN A
TUMOR 15091a ON HOMOIOTRANSPLANTS
OF TUMOR 15091a*

SUBSTANCE INJECTED	HOSTS DYING WITH TUMORS†			
	C57 black-6		C57 brown-cd	
	No.	Per cent	No.	Per cent
Lyophilized tumor 15091a	78/99	79	30/40	75
Saline (controls)	0/34	0	0/13	0
Nothing (controls)	0/54	0	0/20	0

* Summarized data from experiments other than those presented in Tables 1 and 2.

† Numbers in the numerators are the number dying with tumors; numbers in the denominators are the total animals in each experimental group.

be felt, which continued to increase in size until the animal died 16 weeks after inoculation. Occasionally, tumors in the longer-lived animals reached enormous sizes. In one mouse, which was killed for autopsy 11 weeks after inoculation, the tumor mass weighed 17.4 gm., while the animal, minus the tumor, weighed 25.5 gm. The tumor mass was necrotic at the center. There were no obvious metastases.

Experiments with tumor E 0771.—The data for the experiment with the C57 black-6 tumor (experiment 4 of Table 1) are presented in Table 4. The first two columns give the lyophilized materials and the total amount injected per mouse.

Column 3 shows the number dying with tumors (in the numerators) and the total number of mice in each experimental group (in the denominators). The last column gives the per cent dying in each group.

The numbers of positive "takes" of the grafts are about equal following the injections of lyophilized liver, kidney, and tumor. Lyophilized spleen had no effect, for an as yet unexplained reason.

The tumor grafts showed slight initial growth (averaging about 3×10 mm. in width and length) before they regressed completely in the controls and in the group receiving lyophilized spleen. No tumors were found in any of these mice 25 days after inoculation. In animals with positive growths, many of the tumors reached tremendous

TABLE 4

THE EFFECT OF PRIOR INJECTIONS OF LYOPHILIZED TISSUES FROM STRAIN C57 BLACK-6 MICE ON HOMOIOTRANSPLANTS OF THE STRAIN C57 BLACK-6 TUMOR E 0771 IN B ALB C MICE

LYOPHILIZED SUBSTANCE INJECTED	TOTAL AMT. PER MOUSE	HOSTS DYING WITH TUMORS*† No.	Per cent
Normal liver	50 mg.	18/20	90
Normal kidney	"	18/20	90
Tumor E 0771	"	16/20	80
Normal spleen	"	0/10	0
Control (0.85 per cent saline)	5.0 ml.	0/10	0

* Numbers in the numerators are the number dying with tumors; numbers in the denominators are the total animals in each experimental group.

† Tumor E 0771 grows progressively in 100 per cent of C57 black-6 mice. All of 10 C57 black-6 control mice for the above experiment died with tumors.

sizes, often appearing to be as large as the mouse. Central necrosis and erosion of the tumors and the overlying skin were common. Most deaths occurred between the fifth and twelfth weeks after inoculation. This is in contrast to the growth in C57 black-6 mice (in which the tumor is indigenous), where most deaths take place around the third and fourth weeks after grafting. In two animals, one receiving lyophilized liver and the other lyophilized tumor, initial growth of the tumor first was evident in the ninth and tenth weeks, respectively, after inoculation. At this time the animals were about 5 months old. Both of these died with large tumor masses 7 weeks after the first growth appeared (16 and 17 weeks after inoculation). It is interesting that in both cases the growths were located in the left, dorsal posterior regions, rather than over the shoulders where the inoculum was presumably first placed.

DISCUSSION

These experiments show that the processes which lead to a breakdown of resistance in the host to a "foreign" tumor graft can be initiated by both neoplastic and normal lyophilized tissues. Apparently, then, whatever materials initiate these processes are present in both classes of tissues and are not a property of neoplasia *per se*.

It is most likely that the so-called "XYZ" factor, described by Casey *et al.* (1, 2) for tumors in both rabbits and mice, is an expression of the same group of phenomena found in our experiments. The finding of Casey (1) and Drysdale and Casey (3) that normal rabbit testis and spleen extracts had no effect on the growth of the Brown-Pearce tumor may very likely be ascribed to the genetic differences between the tumor and the testes and spleen. This supposition is supported by Casey's observations (1) that injections of frozen Brown-Pearce tumor into mice did not lead to enhanced growth of two mouse tumors tested, Bashford carcinoma 63 and Sarcoma 180. Likewise, injections of frozen Bashford carcinoma 63 had no effect on grafts of the Brown-Pearce tumor in rabbits or on Sarcoma 180 in mice, nor was growth of the Brown-Pearce tumor in rabbits affected by injections of a rabbit adenoma. Snell, Cloudman, *et al.* (9) have similarly recorded the absence of cross reactions, using a variety of mouse tumors and inbred strains of mice.

Our findings and those of Casey raise a number of problems. One of these is inherent in any work involving the tumor transplant technic as a tool for differentiating between neoplastic and normal growth. It is not known whether the events observed in a particular experiment are related to properties which are to be specifically ascribed to neoplastic tissues, as differentiated from normal tissues, or whether they are the result of basic similarities or differences between the host and the implant, be it a tumor or normal tissue. We believe that the phenomena observed are most likely an expression of processes involving compatibility or incompatibility between host and graft, and that these relationships operate for both neoplastic and normal grafts. Such a conclusion is supported by the large mass of data which show that the same general laws of genetic relationship determine the fate of normal and tumor grafts, a point stated in the introduction to this paper. This thesis is to be tested in studies of the effects of lyophilized tissue injections on normal tissue grafts, and on normal growth as exemplified by wound healing.

Other questions to be investigated are (a) the specificity of the effects—that is, the extent to which they are species-specific and inbred strain-specific; (b) the biological basis for the effects; and (c) whether the manner in which the tissues are treated (i.e., freezing, lyophilization) is a necessary prerequisite for effectiveness. Preliminary re-

ports dealing with points (*b*) and (*c*) have been presented by us (5, 6).

That there is a species specificity and even a specificity within the species (genetic specificity?) has already been demonstrated (1, 9). We have also found[1] that lyophilized liver and kidney from guinea pigs, hamsters, and rats, and lyophilized normal rabbit serum did not enhance growth of the strain A tumor 15091a in C57 black-6 mice. The problem of genetic specificity within the species is being further investigated in this laboratory by the use of a variety of inbred strains of mice.

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[1] N. Kaliss, unpublished data.

The Effect of Tumors on Antibody Levels in Mice[*]

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The study of the phenomena of immunity in the presence of neoplastic diseases not associated with demonstrable viruses has been concerned largely with the susceptibility of animals to transplantable tumors of various kinds and with attempts to induce a state of immunity. Little consideration appears to have been given to the possible effects of tumors upon the antibody-forming mechanism or on the antibodies themselves. That a damaging effect may be produced by tumors upon the defense mechanism of the host was suggested by the work of Fleisher and Loeb (3, 4), who found that mice bearing spontaneous tumors were much more susceptible to transplants of tumors from mice of an alien strain than were normal mice. Unfortunately, the authors' use of heterogeneous strains of mice, coupled with the increasing emphasis given to the genetics of tumor development, detracted from the significance of these findings; and no work along these lines appears to have been done until that undertaken by Blumenthal (1), who confirmed the results of Fleisher and Loeb with inbred mice. More recently, Browning (2), using transplanted tumors, and Gorer (5), using induced tumors, have observed that tumor-bearing mice were more susceptible to bacterial infection than normal mice. These findings indicate a breakdown of the host's resistance but give no information on the nature of the change responsible for the lowering of that resistance. The present data show that with certain tumors a loss of antibody occurs.

MATERIALS AND METHODS

The effects most intensively studied were those produced by Sarcoma 37 in Swiss mice and by spontaneous mammary carcinomas in C3H mice.

Additional corroborating data with spontaneous mammary tumors were obtained with dba, Swiss, and A mice, and with other tumors in still other strains. Their sources were as follows: C3H mice with the mammary carcinoma C3HBa obtained from Dr. Morris Barrett of the National Cancer Institute; a transplantable mammary carcinoma (TMC-1) of our own in C3H mice; Sarcoma 180 of the strain used by Dr. K. Sugiura and employed at this Institute by Dr. Theodore Hauschka, in Swiss mice; Earle's sarcoma L obtained from Dr. Glenn H. Algire of the National Cancer Institute, in C3H mice; and a methylcholanthrene-induced fibrosarcoma developed at this Institute by Dr. Elizabeth U. Green in C3H mice.

The transplanted tumors were all implanted subcutaneously in a latero-dorsal position into 8-week-old mice of the appropriate strain and appeared to be growing vigorously when immunizing injections with the various nontumor antigens were begun on about the twelfth day after transplantation. At that time the tumors were generally 1.25 cm. or more in diameter; smaller ones were discarded. The immunized mice were bled from the heart on the eighth to the eleventh day following the last injection of antigen, and the sera were pooled before testing. Tumors of the spontaneous group were single or multiple and usually well grown, and animals bearing such tumors were immunized and bled in the same way as mice with transplants.

A preparation of polysaccharide (P-10) from *S. marcescens*, supplied by Dr. Murray J. Shear of the National Cancer Institute, was the antigen most frequently used. The mice that survived the initial toxic action of the polysaccharide were usually active and apparently well nourished at the time of bleeding. Mice in which the tumors had regressed were placed in a separate group. The immune responses to the polysaccharide were measured by microscopic agglutination tests made with a suspension of *S. marcescens* as antigen (11) and also by quantitative precipitin tests. Because of the small amounts of sera obtainable from mice, the quantitative precipitin tests were carried out

on only 1-cc. aliquots, as follows: To 1-cc. samples of antiserum were added 1-cc. portions of saline containing 10–400 μg. of polysaccharide. The mixtures were incubated at 37° C. for 2 hours, allowed to stand at 5° C. for 2 days, and centrifuged. The precipitates were washed twice with saline and analyzed for total nitrogen by the micro-Kjeldahl method. In control tests it was found that the accuracy of the method was, on the average, within ± 8 μg. of nitrogen.

Human plasma, sheep erythrocytes, and egg white were also used as antigenic agents. Ring tests for precipitin were made when human plasma was used; hemolytic tests, when sheep erythrocytes were used; and both ring tests and quantitative precipitin tests, when egg white was used. Separate controls were run with each experiment.

EXPERIMENTAL

Antibody titers in mice with transplanted sarcomas.—Observations on the active immunization with P-10 of mice bearing Sarcoma 37, Sarcoma 180, Earle's sarcoma L, and a methylcholanthrene-induced sarcoma are recorded in Table 1. In these experiments tumor-bearing and nontumor-bearing mice were examined in a large series of tests with different doses of antigenic polysaccharide. It may be seen that the agglutinating titers of the anti-

sera of the Sarcoma 37 mice were invariably one-half or less than one-half of those of the similarly treated normal mice. This difference appeared to depend less on the size of the tumors than on their vigorous growth. Sex did not seem to influence the results.

Similar differences were observed with the sera of Swiss mice bearing Sarcoma 180. Earle's sarcoma L in C3H mice also produced markedly lower antibody titers, and a methylcholanthrene-induced fibrosarcoma in C3H mice yielded varied differences.

The effects of Sarcoma 37 were confirmed repeatedly in a total of 11 series of tests with 214 tumor-bearing Swiss mice and 170 controls. To put the results on a still surer basis, however, quantitative precipitin tests were carried out. For these tests Swiss mice that had received implants of Sarcoma 37 about 10 days previously, with tumors 1.5 cm. or more in diameter, were injected with 20 μg. of polysaccharide, 3 days later with 100 μg. and were bled 8 days after the last injection. Normal animals were given the same treatment. Complete precipitation was obtained when 200 μg. of polysaccharide in 1 cc. of saline was added to 1-cc. portions of the sera. The amounts of antibody nitrogen in the immune sera of the tumor-bearing mice were consistently found to be only

TABLE 1

AGGLUTININ TITERS OF ANTI-POLYSACCHARIDE SERA FROM MICE WITH SARCOMA 37, SARCOMA 180, EARLE'S SARCOMA L, A METHYLCHOLANTHRENE-INDUCED SARCOMA, AND NORMAL MICE*

	No. of mice	Sex	Age of tumor (days)†	Size of tumor (cm.)‡	Treatment§	1:32	1:64	1:128	Titers 1:256	1:512	1:1024	1:2048
S-37	18	♂	12	1.25–3.2	10(3) 100(3) 200(b10)				+	±	−	−
	13	♂			"				+++	++	+	±
S-37	17	♂	14	1.5–3.25	20(3) 200(b10)	+++	+++	++	+±	±	±	
	14	♂			"			++++	++±	+±	±	
S-37	5	♂	13	2.8×2.0, 2.5×3.0, 3.0×2.1, 1.6×1.5, 2.0×1.8	20(2) 20(b10)			+++±		++	+	±
	8	♀			"			++++		+++±	++±	++
S-180	4	♀	14	2.5×2.7, 2.5×2.7, 1.5×1.6, 1.0×1.0	20(3) 20(b9)			+++	++	±	±	−
	8	♂			"			++++	+++	+±	±	
Earle	4	♂	16	(2.5×2.2, 1.5×1.5) (6.0×2.1, 1.2×0.9) 3.2×2.0, 3.0×2.4	20(3) 20(b9)			+++		++	±	−
MeC	8	♀	13	1.5–3.0	"			+++±		+++±		
MeC	6	♂	15	(2.2×1.3, 2.2×2.0) 3.1×1.8, 3.2×1.6 (2.0×1.7, 2.2×1.9) 3.3×2.3, 3.3×1.9	"			+++		+	−	−
	8	♂			"			++++	+++±	++±	+	

* Eight weeks old at beginning of experiment.

† At beginning of immunization.

‡ At time of bleeding. Sizes are given in terms of range of diameters, or of diameters of individual tumors in two dimensions. Dimensions given in parentheses represent multiple tumors in one animal.

§ Sizes of doses, intervals between injections, and times of bleeding, are recorded as a sequence of figures. For example, 20(3)20(b9) indicates that two injections of 20 μg. each were given at a 3-day interval and the animals were bled 9 days after the last injection.

about one-half as great as in the immunized non-tumor mice (Table 2).

The possibility that the toxic action of the polysaccharide might yield results of limited applicability, and the advisability of testing different

TABLE 2

ANTI-POLYSACCHARIDE N PRECIPITATED FROM IMMUNE SERA OF MICE BEARING SARCOMA 37 AND NORMAL MICE

EXP. NO.	NO. OF MICE S-37	NO. OF MICE Normal	μG. OF PRECIPITIN N/CC OF SERUM Sarcoma 37	μG. OF PRECIPITIN N/CC OF SERUM Normal	PER CENT SARCOMA 37/ NORMAL
1	35	40	57	98	58
2	20	30	47	90	52

TABLE 3

PRECIPITIN LEVELS VERSUS HUMAN PLASMA AND EGG WHITE IN SERA OF IMMUNIZED MICE BEARING SARCOMA 37 AND NORMAL MICE

No. of mice	Sex	Range of diameter of tumor (cm.)	Treatment*	Titers 1:5	1:10	1:20	1:40	1:80
6	♀	1.7–3.5	Human plasma 0.1(2)0.2(3)0.2(3)0.2(b8)	±	–	–	–	
13	♂		"	+++	+±	+	±	–
13	♀	1.8–3.3	Egg white 0.1(1)0.2(3)0.2(b8)	–		–		–
25	♀		"	++±		+		±

* The human plasma and egg white were used undiluted for injections.

TABLE 4

HEMOLYSIS OF SHEEP ERYTHROCYTES BY SERA OF IMMUNIZED MICE BEARING SARCOMA 37 AND NORMAL MICE

No. of mice	Sex	Range of diameter of tumor (cm.)	Treatment*	Titers† 1:800	1:1600	1:3200	1:6400	1:12800	1:25600
9	♀	2.2–4.1	0.2(2)0.25(3)1.0(3) 0.5 (undiluted) (b8)	++++	+++±	++±	–	–	–
8	♀		"	++++	++++	+++±	++±	++±	+

* Washed cells were used in 1:10 dilution except where indicated otherwise.
† Lytic system: Mouse antiserum (dilute), 0.25 cc. Sheep cells 1:100, 0.25 cc. Guinea pig serum 1:10, 0.1 cc.

types of serological reactions, led to the use of human plasma, egg white, and sheep erythrocytes as antigenic agents. The results, shown in Tables 3 and 4, were entirely in accord with those obtained with the P-10 polysaccharide. The findings with egg white were supported by quantitative precipitin tests, which showed that there was as little as 40 per cent as much antibody nitrogen in the sera of tumor mice as in those of the normals.

Since immune responses in other species have been found by electrophoretic tests to be accompanied by increases in serum γ-globulin (6), similar tests were made of the sera used in the present study. Sera were examined from four different groups of Swiss mice: normals, Sarcoma 37 tumor-bearers, immunized normals, and immunized Sarcoma 37 tumor-bearers. Immunization was accomplished by injecting 20 μg. of polysaccharide, followed by a second injection of 100 μg. of polysaccharide 3 days later; the mice were bled 8 days after the second injection. Pools of blood from nine or or more mice were used. The normal variability which might be expected from different pools was established by examination of three different pools of normal sera. Representative electrophoretic patterns are shown in Chart 1, and the actual distributions of protein components, calculated from the diagrams, are shown in Table 5. Antibody titers for the immune sera are also shown in the table. It may be seen, first of all, that there are no significant differences in the total γ-globulin contents of the four sets of sera, although in further experiments the presence of the antibody in this fraction was established by the quantitative precipitin test applied to isolated γ-globulin from immune sera. However, when the data of Table 2 were expressed as gm protein/100 cc, only 13 per cent of the γ-globulin from immune sera of tumor-bearing mice appeared to be antibody γ-globulin, as compared with 23 per cent in the immune sera from normal mice. Thus, although the difference in antibody γ-globulin contents was nearly two-fold, it represented a difference in total γ-globulin content of only 10 per cent. This difference in total γ-globulin content is within normal variation, as shown by data of Table 5 for different pools of sera from normal mice.

Changes which may be noted in proportions of

serum components other than the γ-globulin will be considered in a later section of this paper.

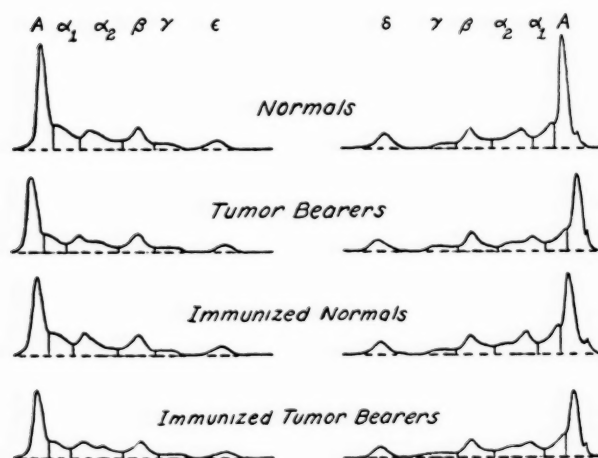*Agglutinin titers in mice with spontaneous and transplanted mammary tumors.*—Antibody titers

CHART 1.—Electrophoretic diagrams of sera of normal and experimental mice. Components were labeled in analogy with components of human serum. Veronal buffer, 0.1 ionic strength, pH 8.6. Left-hand diagrams represent descending boundaries; right-hand, ascending.

were determined in sera from C3H, Swiss, A, and dba mice bearing spontaneous mammary tumors, while normal mice from these strains of the same or approximately the same age served as controls. Except for the Swiss, they were closely inbred. Antibody differences were not, however, found.

These results with spontaneous mammary carcinomas, contrasting as they do with those obtained with the transplanted sarcomas, raised the question whether transplantation might have accounted for the action of the sarcomas. Accordingly, a recently transplanted mammary adenocarcinoma from our own C3H mice was studied, and it was found that mice that had received implants of the 1st, 2d and 5th transplant generations of this tumor (TMC-1) yielded antibody titers equal to those of nontumor-bearing controls (Table 6). This showed that in these generations the effects of transplantation, if any, upon antibody were below the level of sensitivity of the test. On the other hand, mice with the 13th and 17th generations of this tumor, and also mice with transplants of C3HBa, had a much lower antibody titer than nontumor-bearing mice. Thus, although the spontaneous mammary adenocarcinomas

TABLE 5

DISTRIBUTION OF PROTEINS IN SERA OF MICE BEARING SARCOMA 37 AND IMMUNIZED WITH POLYSACCHARIDE

SOURCE OF SERA	DISTRIBUTION OF PROTEINS (GM/100 CC OF SERA)					AGGLUTINATION TITERS				
	Albumin	α_1	α_2	β	γ	1:128	1:256	1:512	1:1024	1:2048
Normals	2.23	0.71	1.02	0.73	0.22					
"	2.56	0.69	1.00	0.76	0.23					
"	2.39	0.79	1.07	0.82	0.27					
Tumor-bearers	1.95	0.55	0.97	0.73	0.28					
Immunized normals	2.00	0.71	1.03	0.80	0.24	++++	+++±	+++	++±	+±
Immunized tumor-bearers	1.71	0.52	1.02	0.70	0.26	+++±	++±	++	+±	±

TABLE 6

AGGLUTININ TITERS OF ANTI-POLYSACCHARIDE SERA FROM C3H MICE WITH TRANSPLANTABLE MAMMARY TUMORS

No. of mice	Sex	Age (mo.)	Kind of tumor	Generation of tumor	Age of tumor (days)	Range of diameter of tumor (cm.)	Treatment	Titers				
								1:128	1:256	1:512	1:1024	1:2048
2	♂	3	TMC-1	1	32	1.0–2.8	20(3)20(b8)		++++		+±	
6	♂	3					"		+++±		++	
10	♀	3	TMC-1	2	22	1.5–3.5	20(3)20(b11)		+++±	++±	+±	±
5	♀	3	"	2	22	1.3–3.5	"		+++	++±	+±	±
6	♂	3	"	2	22	1.3–2.5	"		+++	++	+±	±
3	♂	3					"		+++±	+++±	±	±
4	♂	4	TMC-1	13	16	1.6–4.5	20(3)20(b9)	+++±	+++	+++	+±	±
7	♂	4					"	++++	++++	+++	++±	+±
6	♂	4	TMC-1	17	15	1.5–5.4	20(3)20(b10)		+++	++	±	±
8	♂	4					"	++++	+++±	++±	+	
5	♂	4	C3HBa	61	16	1.3–3.0	20(3)20(b9)	+++±	+++	++	+±	+
7	♂	4					"	++++	++++	+++	++±	+±
2	♂	3	C3HBa	49	14	1.5–3.5	20(3)20(b11)		++	+	−	−
4	♀	3	"	49	14	3.5+	"		++	±	−	−
7	♀	3	"	49	14	3.5+	"		+±	+	±	−
3	♂	3					"		+++±	++±	+	±

failed to bring about a lowering of the antibody titer, two transplanted mammary adenocarcinomas were capable of doing so. This means, therefore, that the antibody effect is not limited to sarcomas.

It appears that the ability of TMC-1 to affect the antibody level depends on its rate of growth (Table 6). TMC-1 grew relatively slowly in the first generations, while by the 13th generation it was growing as rapidly as the well established and fast growing C3HBa.

Effect of regression on antibody titers.—The tendency of Sarcoma 37 to regress spontaneously in a small percentage of mice, and with greater frequency in mice treated with bacterial polysaccharide, presented the opportunity to assay the antibody levels in the sera of mice whose tumors had regressed. It was found by agglutinin and quantitative precipitin tests that the titers of the sera of both groups of mice were much higher than those of mice bearing vigorously growing tumors and approximated very closely those of similarly treated normal mice. The same type of result was obtained with mice bearing regressed tumors when immunization was carried out with human serum or sheep erythrocytes.

A parallel effect in mice bearing slowly growing tumors was also observed. In the previous studies the tumors with which the phenomenon of antibody differences was demonstrated were about 2 weeks old at the beginning of immunization and about 1.25 cm. in size or larger. Such tumors, in untreated mice, usually continued to grow rapidly, so that at a period corresponding to the time of bleeding they were 2.5–3 cm. or larger. There was little or no difference in the antibody titers of mice whose tumors grew more slowly than this, when compared with similarly treated normals.

The rate of growth, rather than the size of the tumors, appeared to be the determining factor in causing lowered antibody levels, since the relatively slowly growing spontaneous mammary tumors, even when very large, and the tumor TMC-1, in its early transplant generations when growing slowly, consistently failed to show this effect.

Possible sources of serum antibody changes.— Lowered antibody titers may be due to any of the following: (*a*) the tumor may have destroyed or modified the antibody after it was formed; (*b*) it may have produced an inhibitor that interfered with the serological reactions; (*c*) it may have so changed the protein composition of the serum as to alter the optimal physical conditions for the serological reactions to take place; or (*d*) it may

have damaged the antibody-forming mechanism of the host.

The question of possible antibody destruction was studied by means of a passive transfer of serum from immunized normal Swiss mice into normal mice or mice bearing Sarcoma 37 or Sarcoma 180. P-10 antiserum from normal mice was injected intraperitoneally into sarcoma-bearing and normal mice, which were bled 4 days later and then tested for antibody titers (Table 7). Titers in the tumor-bearing animals were again lower than in the similarly treated normal controls. These results indicate that, apart from whatever action the tumors may have had upon antibody production, formed antibody was destroyed in the tumor mice more rapidly than in the normals. Incidentally, rabbit P-10 antiserum failed to show a difference in titer after being injected into normal and tumor-bearing mice (Table 7).

To observe whether antiserum from tumor-bearing mice continued to lose antibody *in vitro*, antiserum of known titer from tumor-bearing animals was held for several hours at 37° C. and was then retested; however, no change in titer was indicated. Heating the serum at 56° C. for $\frac{1}{2}$ hour failed to change the agglutinating titer; refrigeration at 5° C. for several weeks also left it relatively unchanged. It appeared from this that there was no continuing destructive effect of storage on the serum *in vitro*.

In attempting to demonstrate an inhibitory effect *in vitro*, anti-P-10 serum from immunized normal mice was titrated in the presence of serum from nonimmunized Sarcoma 37 mice. The Sarcoma 37 serum was diluted 1:9 before use as the diluent, and normal mouse serum, diluted 1:9, served as the control diluent. In other experiments, serum from nonimmunized tumor mice was added to anti-P-10 serum from normal mice in higher proportions, namely, 2:2 and 1:3, and incubated at 50° C. for 15 minutes prior to titration; corresponding controls were run with normal serum replacing Sarcoma 37 serum. It was found that the serum from the Sarcoma 37 mice failed in all instances to inhibit the agglutinating activity of antiserum from normal, i.e., nontumor-bearing mice.

That serum protein levels might contribute to low antibody titers is suggested by the relatively low levels of fraction a_1 and albumin in the serum of tumor-bearing mice (Table 5). This finding is consistent with the low albumin levels which are common in human cancer (7–10). Nevertheless, certain considerations prevented acceptance of this relationship as one of cause and effect. If a lowering of one or more serum proteins accounted for

the inability of the antibody to exhibit maximum titer, then, by supplying the deficiency, the differences in titer should disappear. However, when antisera from tumor-bearing and control mice were titrated in normal serum diluted 1:100, there was no change in the relationship of the titers. Dilution of the antisera 1:7 with normal serum prior to dilution in saline similarly failed to change the ratio of the titers.

The antibody-forming mechanism of the host was not tested directly. However, the ability of

in tumor-bearing mice is highly significant for any concept of immunity in the neoplastic state, inasmuch as it establishes the lessening of specific immune reactions which may be of importance to the growth of tumors themselves. The fact that an excessive loss of antibody protein is the essential feature of this phenomenon makes it pertinent to the problem of tumor management and therapy. If we may assume that the antibody-forming mechanism of the host has not been irreversibly affected by the growth of the tumors studied, it would seem

TABLE 7

AGGLUTININ TITERS VERSUS *S. marcescens* OF SERA FROM TUMOR-BEARING AND NORMAL 8-WEEK-OLD SWISS MICE PASSIVELY IMMUNIZED WITH ANTISERUM FROM THE MOUSE OR RABBIT

No. of mice	Kind	Tumor Age (days)	Size (cm.)	Treatment	Titers 1:64	1:128	1:256	1:512	1:1024
3	S-180	21	2.0–3.0	Mouse a.s. 0.5 cc.	+++	+±	+	±	
4				"	+++±	++±	++	±	
7	S-37	18	1.2–3.3	Mouse a.s. 0.5 cc.	++	+±	±	−	±
7				"	+++±	++±	+±	+	±
8	S-37	14	1.25–3.0	Rabbit a.s. 0.5 cc.	++±	++±	+±	+	±
7	S-37	14	1.25–3.1	"	++±	++±	+±	+	±
10				"	++±	++±	+±	+	±

mice whose tumors have regressed (whether prior to or during immunization) to produce antibody titers equal to those of normal mice indicates that, if there was damage to the antibody-forming mechanism, it was not permanent.

DISCUSSION

The quantitative differences between the antibody levels in immunized normal and tumorous mice suggest that, if γ-globulin is the sole locus of antibody, either (*a*) there is a selective use or destruction of antibody γ-globulin or (*b*) the replacement of destroyed or utilized γ-globulin takes place unequally between nonantibody γ-globulin and antibody γ-globulin.

Although a corresponding weakening of antibody has not been demonstrated in mice with spontaneous mammary tumors, the findings of Fleisher and Loeb (3) and of Blumenthal (1), that mice with spontaneous tumors are more susceptible than normal mice to transplants of tumors from heterologous strains, indicate that an adverse effect on antibody action by these tumors may yet be found. This effect may possibly be of the same kind as that we have observed but of a different magnitude. The lower antibody content of mice with transplanted spontaneous mammary tumors, as shown with C3HBa and later generations of our TMC-1, indicates that, under favorable conditions of growth, carcinomas also are capable of modifying the antibody status of the host.

The phenomenon of lowered antibody activity

that antibody production could be made to compensate for, and overcome, the tendency toward decreased effective antibody, to the ultimate advantage of the host.

SUMMARY

As a result of immunization with various antigens, mice with certain transplanted tumors, namely, Sarcoma 37, Sarcoma 180, Earle's sarcoma L, a methylcholanthrene-induced fibrosarcoma, and Barrett's transplantable mammary adenocarcinoma, yielded antisera of a lower titer than those from similarly treated normal mice. Antisera from tumor-bearing mice had one-half, or less than one-half, of the agglutinin titer of antisera from normal mice. In terms of precipitin N, immunized Swiss mice bearing Sarcoma 37 yielded as little as 40 per cent as much antibody as did similarly treated normal mice. Spontaneous mammary tumors did not elicit this effect, but a transplanted mammary tumor, TMC-1, acquired the property of doing so after thirteen transplantations. Mice in which tumors had regressed yielded amounts of antibody equal to those of normal mice. It was concluded that rapidly growing transplanted tumors lower antibody titers, but that slowly growing, or regressed, tumors do not do so.

The phenomenon was also demonstrable by passive transfer of mouse antisera into tumor-bearing and normal mice and was found to be specific, inasmuch as rabbit antisera passively transferred in mice failed to show this difference. This

indicated that an abnormally high destruction of antibody occurred in tumor-bearing mice. The effect did not appear to be due to inhibitors or to changes in other serum components.

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The Effect of Foster Nursing on the Growth of a Transplantable Tumor

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INTRODUCTION

A transplantable mammary adenocarcinoma of mice has been observed to grow at a significantly different rate in reciprocal F_1 hybrid hosts, depending upon which of the two inbred strains used in the matings was that of the mother and which that of the father (2). Some uncertain analogies in the behavior of spontaneous tumors suggested that the influence was derived from the mother and was probably transmitted through the milk, but the available data were not sufficient to support any definite conclusion as to the source or nature of the influence.

This report is concerned with further experiments, performed to determine the effect of foster nursing, which showed that the difference in growth rate observed previously is not present if all the hybrid hosts suckle a mother belonging to the strain of origin of the tumor.

MATERIALS AND METHODS

The methods and materials employed have been described in detail in a previous paper (2), and only the more important points will be given here.

All tumors were grown in reciprocal F_1 hybrids between inbred strains C3H and C (B alb C) from the colony maintained by Dr. W. E. Heston at the National Cancer Institute. As in previous work, the hosts were divided into two groups in each experiment, one group consisting of the C3H $\times$ C F_1 hybrids and the second of C $\times$ C3H F_1 hybrids. In each experiment the animals in both groups were matched for age, sex, and number, so that, although there was some variation between experiments, the comparisons of tumor weights were always made between similar groups.

Growth rates were determined by excising and weighing the tumors individually after a 3-week period; the average tumor weight in each group of a given experiment was then determined and used to calculate the relative growth rate between

classes of animals being compared. Thus, the results are expressed as the value of a ratio, the numerator of which is the average tumor size in C $\times$ C3H F_1 hosts (either foster-nursed or not, as the case may be) and the denominator of which is the average tumor size in C3H $\times$ C F_1 hosts.

Three sets of experiments were performed. The first, comprising fourteen experiments with a total of 420 animals, was a repetition and extension of the earlier work in which none of the animals was foster-nursed. Concurrently, a second set of seventeen experiments involving 346 animals was done; in these the C $\times$ C3H F_1 hosts were foster-nursed (beginning on the day of birth) upon C3H foster mothers), and the C3H $\times$ C F_1 hosts were allowed to suckle their own (C3H) mothers. Subsequently, a third set of three experiments involving 187 animals was done as a further check. In these last experiments, the C $\times$ C3H F_1 litters were separated at birth into two groups, matched as nearly as could be for number and sex. One-half of the litter was allowed to nurse its own (C) mother, and the other half of the litter was foster-nursed by a C3H mother. From these fostered and unfostered litter mates matched groups were made up for experiments in which the tumor growth rate in each was compared with the growth rate in C3H $\times$ C F_1 hosts. In effect, these last experiments recapitulate the two previous series simultaneously and have the additional advantage of comparing litter mates.

All hosts received subcutaneous inoculations of 0.025 cc. of a 50 per cent suspension of the tumor. The tumor, a mammary adenocarcinoma which originated in a C3H female, grows in all strain C3H mice and in all F_1 hybrids, such as were used here, but will not grow in strain C mice. Since the previous communication, we (in collaboration with Dr. H. B. Andervont) have determined, by injecting tumor extracts into strain C females, that the tumor contains the mammary tumor milk agent and retains it when grown in either of the hybrids used here.

Among the minor details of technic there are

two unimportant differences between the present and the previous experiments: namely, the animals were housed in plastic cages, and the age of the hosts ranged from 5 to 17 weeks, but as before most of the animals were between 8 and 12 weeks of age.

RESULTS

The tumors showed the same sort of individual variation in size as was noted previously, and in each class the variation appeared to be randomly distributed about a mean which was different for different classes. In the earlier work there was a trend in the curves shown on the distribution diagrams which suggested that, if a series were carried on by serial transplantation within the hybrid lines, there would be a continuous decline in the average weight of the tumors. It turned out that the trend was apparent and not real, for when one such series was carried to the twenty-first generation there was no general decline in the average weight of the tumors. Since nothing further concerning minor details of the results could be learned from diagrams similar to those originally used, a different and more facile method of presentation has been adopted. In this report all the data are summarized in a single chart. In each experiment (including the eleven reported earlier), the result is expressed as the ratio of the average tumor size in $C \times C3H F_1$ hosts to the average tumor size in $C3H \times C F_1$ hosts. Obviously, experiments in which there is no significant difference in growth rates will yield a ratio that is 1, or nearly 1, and experiments in which there is a difference in growth rates will yield a ratio that is greater or less than 1 according to circumstances.

Chart 1 shows such ratios for 45 experiments involving 1,119 animals. Each point represents the value of the ratio determined in a single experiment (about twenty animals, ten of each kind). In each column a group of ratios are shown for a series of experiments which are related to each other by the serial transfer of the inoculum from hybrid to hybrid. The columns are not directly related to each other, because at the beginning of each series fresh inoculum was taken from the stock tumor. This arrangement has the advantage of guarding against misinterpretation due to unforeseen spontaneous changes either in the stock or in the serial transplants. The first three columns are the results of the previously reported work shown as ratios for comparison with the present work. The fourth and fifth columns are the results of fourteen experiments like the first but performed later and concurrently with the foster-nursing experiments. The five experiments shown in the fifth column differ from the others in that

the animals were allowed to carry their tumors until death, and the tumor sizes were expressed as the product of caliper measurement at 3 weeks, a less accurate but, in this case, acceptable method of measurement. The first five columns of Chart 1, representing all the experiments without foster
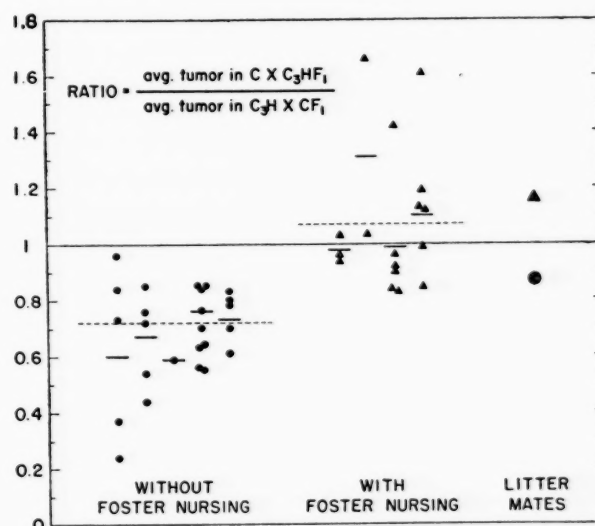

CHART 1.—Distribution diagram showing the numerical value of the ratios between average tumor sizes in reciprocal hybrid hosts with and without foster nursing of the hybrids having a strain C mother. The ratios were computed by the formula shown in the upper left corner in all cases. The value of the ratio would be 1 when the growth rate was the same in both hybrid hosts; the value would be less than 1 when the growth rate was slower in the $C \times C3H F_1$ hybrids and vice versa. Since these are random populations, a value of exactly 1 would not be expected except in extremely large samples. Each point (except the two at the extreme right) represents the result in a single experiment with about twenty animals, ten of each maternal ancestry. A series of experiments related to each other by the serial transfer of tumor from hybrid to hybrid is shown in each column, and the average ratio for that series is shown by the solid line in that column. The columns are not related to each other directly and represent repetitions of the work. The dashed lines show the over-all ratios for all experiments with and all experiments without foster nursing. The two large symbols at the right show the over-all ratios obtained in three experiments using matched litter-mate groups: with foster nursing, the triangle; and without foster nursing, the dot.

nursing, show that the ratios for all experiments are less than 1; the averages within the columns vary from 0.59 to 0.76 (these are calculated from the aggregated tumor weights and are not the simple arithmetical averages of the ratios), and the ratio obtained by considering all 586 tumors together is 0.72. It is quite obvious that the true value of the ratio in this group is near 0.7, which is to say that the tumors in the $C \times C3H F_1$ hosts were smaller than those in the reciprocal hybrid. Statistical analysis indicates that the difference is highly significant (P is much less than 0.001).

Columns 6–9 of Chart 1 show a striking contrast to the previous results. These four columns show the ratios obtained in a set of seventeen experiments, done in four series, involving a total of 170 C × C3H F₁ hybrids foster-nursed on strain C3H mothers and 176 C3H × C F₁ hybrids that nursed their own (C3H) mothers. In these experiments the differences observed previously are not present. The values for the ratios between average tumor weights are randomly distributed about a value near unity, and there is no value for a single experiment as low as the mean value in the work with fostered hosts. The over-all ratio obtained by aggregating all tumors in these experiments is 1.07 and is a near approach to unity, which is to say that the growth rates in the two reciprocal hybrids were approximately equal under these conditions.[1]

The last column in Chart 1 shows the ratios obtained when tumors in fostered and unfostered litter-mate C × C3H F₁ hybrids were simultaneously compared with tumors in C3H × C F₁ hybrids. For the sake of simplicity, only the ratios derived from over-all averages are shown. Actually, three such experiments with a total of 187 animals were done, and in each experiment the ratio for tumors from fostered hosts was well above that for tumors from unfostered hosts. The tri-

[1] The authors gratefully acknowledge the assistance of Mr. Jerome Cornfield, Biometrics Section, National Cancer Institute, who made all of the statistical analyses.

With regard to the value of the over-all ratio in the experiments employing foster nursing, it should be noted that the value 1.07 given above is derived by a simple method, which makes no allowance for the varying reliability of the several estimates of the mean derived from random samples of the population. The likelihood that the true value of this ratio is unity is a point of some theoretical interest. Accordingly, Mr. Cornfield has used more precise methods to calculate that the value of the ratio is 1.040 ± 0.033.

Each of the seventeen experiments yielded an independent estimate of the ratio. These estimates vary in reliability, however, owing to differences in the number of animals used and to differences in the variation in weight of the individual tumors within a single experiment. In consequence, the over-all ratio was computed as a weighted average of the individual ratios, the weighting factor being the reciprocal of the sampling variance of the ratio for each experiment. The sampling variance was estimated for each experiment by means of the formula:

$$\text{variance} = \frac{S^2}{M^2}\left[1/n_1 + 1/n_2\right],$$

where S is the pooled standard deviation, M is the pooled mean tumor weight and n_1 and n_2 are the numbers of animals in each of the two classes. The standard error of a weighted average so computed is estimated as the reciprocal of

$$\sqrt{\sum \frac{M^2}{S^2} \frac{n_1 n_2}{n_1 + n_2}},$$

the summation extending through all seventeen experiments.

angle represents the ratio obtained when foster-nursed C × C3H F₁ animals were used (as in columns 6–9), and the dot shows the ratio obtained without foster nursing (as in columns 1–5).

The average tumor weights from which the ratios shown on the chart were computed are given in Table 1. Both the values for single experiments and the aggregated values for each series are shown. It may be repeated that the over-all ratios are not arithmetical averages of ratios but were calculated by aggregating all the individual tumors involved. To give all the individual weights for 1,119 tumors would require too large a table, and this has been omitted. The random distribution of individual weights was similar throughout the work, and this can be seen by referring to the diagrams in the earlier publication where the first eleven experiments were reported.

DISCUSSION

The results of the experiments done without foster nursing confirm the existence of a "maternal influence" on the growth rate of this tumor and, from a statistical standpoint, place it beyond question. It is equally clear that when foster nursing is done in the manner described one may observe an effect which points to the milk as a source of the major, if not the only, factor in this phenomenon.

The interpretation of these findings which seems most attractive at the moment is that the growth rate of this tumor may be affected by the presence or absence of the mammary tumor milk agent in the secondary host, although the tumor originated in the presence of the agent and contains the agent within its own tissues. However, this interpretation is by no means certain; it may be that some factor in the milk other than the mammary tumor milk agent is responsible for the results reported. Further experimentation will be required to settle this point. The possibility that the foster mother exerts some influence of unknown nature outside those in the milk is not excluded, but it does not seem likely.

It should be emphasized again that the results reported herein have to do with the rate of growth of the tumor as measured by the weight attained in 3 weeks, and they do not reflect the transplantability as measured by the number of "takes." As has been said before, the tumor "takes" in all these F₁ hybrids, regardless of the maternal parentage and regardless of foster nursing. In this respect, these results agree with those of Bittner (3), who reported that in F₁ hybrids the transplantability (as measured by "takes") of a series of tumors did not depend upon the presence (or source) of the milk agent in the hosts. However, at

TABLE 1

THE EFFECT OF FOSTER NURSING ON THE GROWTH OF A TRANSPLANTABLE TUMOR

Exp. no.	Av. wgt. of tumors at 3 weeks in C×C3H F1 hosts (gm.)	in C3H×C F1 hosts (gm.)	Ratio C×C3H F1 to C3H×C F1	Totals in C×C3H F1 Animals	Weight of tumors (gm.)	Totals in C3H×C F1 Animals	Weight of tumors (gm.)	Ratio of average tumor weights	Strain of mother suckled by C×C3H F1 hybrid hosts*
Series I:									
No. 1	2.28	6.13	0.37						
2	4.23	5.81	0.73		109.4		207.4		
3	4.14	4.94	0.84	35		40		0.60	C
4	3.95	4.11	0.96		(3.13 av.)		(5.19 av.)		
5	0.92	3.68	0.25						
Series II:									
No. 1	6.72	7.88	0.85						
2	6.95	9.66	0.72		201.7		269.6		
3	6.03	7.96	0.76	39		35		0.67	C
4	3.29	5.86	0.56		(5.17 av.)		(7.70 av.)		
5	2.78	6.43	0.43						
Series III:									
No. 1	3.99	6.76	0.59	9	35.9	8	54.1	0.59	C
Series IV:									
No. 1	5.03	5.90	0.85						
2	4.89	7.73	0.63						
3	4.15	5.94	0.70						
4	2.95	5.24	0.56		595.3		938.9		
5	2.73	3.21	0.85	141		170		0.76	C
6	4.26	5.64	0.76		(4.22 av.)		(5.52 av.)		
7	3.06	4.79	0.64						
8	4.50	5.35	0.84						
9	3.74	6.77	0.55						
Series V:									
No. 1	1.78†	2.51†	0.71						
2	4.45†	5.59†	0.80		161.2†		249.4†		
3	2.52†	4.10†	0.61	51		58		0.73	C
4	4.18†	5.34†	0.78		(3.16 av.)		(4.30 av.)		
5	5.19†	6.19†	0.83						
Series VI:									
No. 1	1.37	1.33	1.03		67.7		68.9		
2	3.48	3.63	0.96	24	(2.82 av.)	24	(2.87 av.)	0.98	C3H
3	4.37	4.64	0.94						
Series VII:									
No. 1	6.00	3.61	1.66	16	105.8	20	100.6	1.31	C3H
2	7.39	7.16	1.03		(6.61 av.)		(5.03 av.)		
Series VIII:									
No. 1	3.84	4.18	0.92						
2	8.01	8.35	0.96						
3	6.98	7.60	0.92		400.3		427.6		
4	9.89	6.98	1.42	55		58		0.99	C3H
5	7.53	8.92	0.84		(7.28 av.)		(7.37 av.)		
6	7.65	9.22	0.83						
Series IX:									
No. 1	6.09	7.19	0.85						
2	6.37	5.36	1.19						
3	7.66	6.80	1.13		638.0		574.0		
4	11.11	6.90	1.61	75		74		1.10	C3H
5	10.33	10.39	0.99		(8.51 av.)		(7.76 av.)		
6	10.45	9.36	1.12						
Series X:									
No. 1a	5.33	6.67‡	0.80	69	330.0	58‡	319.0‡	0.87	C
2a	4.59	4.36‡	1.05		(4.78 av.)		(5.50 av.)		
3a	4.71	5.92‡	0.80						
1b	7.51	6.67‡	1.13	60	384.3	58‡	319.0‡	1.16	C3H
2b	5.37	4.36‡	1.23		(6.41 av.)		(5.50 av.)		
3b	6.67	5.92‡	1.13						

* All the C3H × C F1 hybrids suckled their own C3H mother.

† Size measured in square cm. in these experiments. Not used in computing final ratios. See text.

‡ These figures are duplicated in the table because the "a" and "b" parts were done simultaneously and the fostered and unfostered groups were both compared with the same group of C3H × F1 hosts. See text.

the present time it does not seem safe to generalize regarding possible maternal influence on the "take" of a tumor, because Cloudman (4) and Law (5, 6) have reported that under other circumstances foster nursing increased the percentage of successful inoculations of a graft. Furthermore, in work reported elsewhere (1) we have made observations which, although of borderline significance statistically, suggested that the tumor used here "took" better in backcross (to resistant) mice having a strain C3H maternal grandmother than in those having a strain C maternal grandmother. This apparent conflict between results which probably are correct individually (special conditions being required for each) might be explained by assuming that several factors are involved, and the more subtle influences will be seen only when they are not overwhelmed by more potent ones.

The experiments reported thus far do not permit analysis of the data in search of effects due to the minor differences between reciprocal hybrids. Although such hybrids are in general alike, there are certain well known differences (Russell [7]). Differences due to the opposite origin of the X and Y chromosomes are negated in these experiments by distributing the sexes equally through the groups. This cannot be said of the other differences between the two hybrids, but that would not alter the conclusion that a difference in growth rate of the tumors was present in unfostered hosts and not present in fostered hosts.

SUMMARY

A mammary adenocarcinoma, which originated in a strain C3H female and contains the mammary tumor milk agent, was transplanted into reciprocal F_1 hybrids between strains C3H and C. The tumor grew in all hosts but at a more rapid rate in those having a strain C3H mother than in those having a strain C mother.

When the hybrids having a strain C mother were foster-nursed on strain C3H foster mothers, the difference was no longer present.

The interpretation that the observed effect of foster nursing was due to the mammary tumor milk agent is considered to be possible but remains uncertain.

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The Vascularity of Normal and Neoplastic Grafts *in Vivo**

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The chief purpose of this work was to determine the microscopic appearance and manner of vascularization of living grafts from an undifferentiated epidermoid carcinoma of rabbits, the V2 strain of Kidd and Rous (6), and to compare such information with data already obtained in this laboratory from the study of autogenous grafts of normal tissue.

Two accounts of work done along similar lines have been found in a search of the literature. Ide, Baker, and Warren (5) studied the vascularization of living grafts of Brown-Pearce rabbit epithelioma, using a transparent chamber method. They observed vessel growth around the tumor in from 3 to 8 days after transplanting. Within 24 hours after vessel growth started, the tumor began to grow. They found all the normal mechanisms of vascular growth to be present but observed that the speed of vascular development was much greater in the tumor than in the homologous grafts used as controls. They thought it likely that the tumor "elaborated a vessel growth-stimulating substance."

Using a much more refined chamber method and applying it to mice, Algire and Chalkley (1) studied "the vascular reactions to wounds and to normal and neoplastic transplants." The normal transplants were homogenous. The tumors studied were sarcomas and mammary gland carcinomas. They found that "an outstanding characteristic of the tumor cell was its capacity to elicit continued growth of new capillary endothelium from the host." They believed that such a characteristic is an important expression of neoplastic change.

In this study only autogenous grafts of normal tissue were used for comparison with the neoplastic tissue, because, with the methods used, homo and hetero grafts have not survived for more than a few days or weeks, whereas autografts of many tissues have survived indefinitely. Since the V2

carcinoma also survives readily in transplants, it behaves in that respect like an autograft, despite its homogenous origin. This in itself would seem to be an important difference between the two types of tissue, normal and neoplastic.

METHODS

Living specimens of the tumor were obtained through the kindness of Drs. Lucké and Breedis, Department of Pathology, University of Pennsylvania. The method they used for routine transplanting, to keep the tumor available, was to make an emulsion by pressing it through a fine wire sieve into a dish of salt solution, whereupon the suspension was injected into the hind leg muscles of the host. Injections of 0.5–1 cc. result in tumors that kill the animal in a few weeks, sometimes as few as 6–8. Since it was not known whether or not the tumor would survive in subcutaneous transplants in a restricted space such as the external ear, preliminary experiments were done to determine that point. The six domestic rabbits used for that purpose were about 6 months old, colored, unrelated, and equally divided as to sex. In one ear of each animal numerous subcutaneous tunnels, one end of each being blind, were made between the outside skin and cartilage and about 1 c. mm. of tumor placed in each. When it was demonstrated that the tumor would grow under these conditions, small grafts were then placed in vascularized transparent chambers that had been installed in rabbit ears for about 6 months. The three rabbits used for this purpose were all colored males and about 1 year old when the grafts were made.

The transparent chamber method is one whereby a thin region is created in an animal so that it may be studied repeatedly with the microscope and in other ways. In the present instance a tantalum and mica chamber was used. When completely vascularized, the chamber could be opened. The vessel layer, about 40 μ thick, could be exposed under fluid, and grafts could be made upon it. When the cover was replaced, normal grafts

could be studied microscopically for an indefinite period in the lifetime of the animal. Construction of the chamber and the technic of installation and grafting have already been described (12). All operating and transplanting were done with aseptic precautions. Two tumor grafts were placed on the vessels in each of the three chambers. After compression by the cover slip, these grafts were about 20 μ thick and 300 μ in greatest diameter.

Autografts of normal tissue have been made over a period of many years, in the same general manner, in more than 100 animals. Some of the tissues studied, each of them for many months, are: thyroid, adrenal, testes, lymph node, mammary gland, bone, sympathetic ganglia, parathyroid, fat, spleen, yellow marrow, and epidermis (7–11).

When sections were made, specimens were fixed in Bouin's solution, cut in paraffin at 5–8 μ and stained with hematoxylin and eosin.

OBSERVATIONS

Subcutaneous Grafts of Tumor in Ears

All grafts made subcutaneously in the ears survived and increased in size. Within 5 days definite masses could be seen and felt at the graft sites. These continued to grow and at the end of 4 weeks appeared as in Figure 5. The tumor areas were red from the vessels in them engorged with blood. After 6 weeks tumors began to ulcerate at the most elevated part. Serum oozed from the skin over the tumor, and then the skin rapidly disappeared, leaving a raw, grayish-white surface from which serum continued to ooze (Fig. 8). The margins of the lesion were sharp, and while the tumor was actively growing there was no evidence of healing of the epidermis. If, before a tumor broke down, it was incised full depth in the long axis, the animal being unanesthetized, there was no evidence of pain until skin beyond the tumor was reached. Most tumors so treated erupted a thick necrotic mass, but a few small ones did not, and the cut surface bled but little. These cut tumors became encrusted with dried exudate and were then indistinguishable from those that had not been cut, since all tumors eventually developed an eroded surface.

All tumors continued to extend laterally, not all to the same degree, after erosion of the center, and were more or less circular in shape. The eroded area increased in diameter and was surrounded by a raised indurated region. Ulcerated tumors developed hornlike masses on the surface. These extended as much as 1 cm. above the skin before breaking off. This occurred repeatedly and seemed to be an exaggerated sort of scab formation, the edges of which had the type of serrations found on a ram's horn.

In no case did tumor implantations in the ears alone cause the death of animals. If about the same total volume of tumor as was placed in an ear was implanted into the muscles of the leg of another animal, death ensued in from 6 to 10 weeks. These leg tumors were many times larger than ear tumors at the time of death. When a tumor in an ear reached about the size of those shown in Figure 8, it became stationary, if there were no muscle transplants, and then underwent slow regression. At the end of about 6 months all ear tumors completely disappeared, and the areas that had previously been eroded were covered with epidermis. No metastases were found in any animals, although the tumor does frequently metastasize (6). Since no attempt was made to keep the eroded surfaces sterile, it may be assumed that they were all infected secondarily, a factor that may have been concerned with their disappearance. There was no invasion of the cartilage or extension to the opposite side of the ear.

Since the skin on the outside of the ear, where the grafts were placed, was fairly tightly attached to the underlying cartilage, it was thought that this might have been a factor in the erosion of the skin as the tumor grew. In one animal subcutaneous transplants were made in a leg where the skin was loose. The skin eroded over the tumor in the same way and in about the same time as it did in the ear, but no hornlike masses formed. This animal also contained intramuscular transplants that killed it. There was, therefore, not sufficient time to determine whether or not an eroded skin tumor can heal in the presence of additional tumors within the body. Transplants in muscle, if they kill the animal (this they have done uniformly so far) perform their lethal work well within the 6-month period apparently necessary for a skin tumor in the ear to disappear and the area to heal.

Grafts of Tumor in Chambers

All tumor grafts made into chambers survived and increased in size. When the chamber cover was reapplied after the transplanting operation, grafts were flattened and became so nearly transparent that they could be located only with difficulty. This flattening did not interfere with blood flow in any underlying vessels. Similar treatment of grafts of normal tissue would, in my experience, have resulted in occlusion of some vessels. This suggests that normal tissues have a solidity or turgidity not possessed by the tumor.

The histories of all chambers containing tumor grafts were similar, and each covered a period of

about 30 days after which the contents were so altered that nothing further could be learned from them. However, the tumor continued to grow. There was no evidence of this on the inside of the ear, but over the chamber on the outside a large mass formed, which, when the animal died, measured roughly 3.5 × 3 × 1 cm., a size reached in 9 weeks from grafts the original dimensions of which were about 0.3 × 0.2 × 0.02 mm. The entire center of the tumor was necrotic.

The obverse and reverse of a chamber that had been installed in an ear for 6 months is shown in Figures 1 and 2. Similar views of a chamber into which tumor had been transplanted 9 weeks previously are shown in Figures 3 and 4. The growth around the chamber in Figure 3 is not tumor but a keloid-like formation of connective tissue not associated in any way with the transplanted tumor, since such overgrowth occurs frequently around chambers that do not contain tumor. This growth appears to be related to the degree of compression of the skin and cartilage when the chamber was installed. Although not a part of the present study, this in itself is an interesting cellular response, since it amounts to benign connective tissue tumor formation resulting, in the first instance, from mechanical factors. The mass shown in Figure 4 is a tumor that grew through the chamber from grafts that were placed in the observation space—the central, clear circle in Figure 1. Figures 3 and 4 were taken after fixation.

Increase in the size of the grafts began quickly after they were placed, and within 48 hours the area covered by tumor had increased by about one-third. There was no evidence that tumor cells immediately began invasion of the vascular layer. In all chambers there is a very small amount of fluid covering the vascular layer on both sides and separating it from the mica that bounds the observation space. It was in one of these thin fluid layers that the grafts were placed. Tumor cells grew rapidly into this space, forming a thin sheet over the vessels. This growth was not associated with or dependent upon invasion of tumor grafts by blood vessels.

The layer of tumor cells was composed of bands and whorls of cells, some arranged in an acinar fashion.

Figure 9 shows the vessels immediately after placing the grafts. They occupy the center of the area and, unlike grafts of normal tissue, the edges are not easily seen and the bodies are not sufficiently dense to make them conspicuous. At *X*, a spicule of bone can be seen. This arose spontaneously, as bone sometimes does in chambers, and was present before the transplants were made.

Eight days after grafting, the bone had disappeared (Fig. 13).

Two days after grafting (Fig. 10), the appearance was not greatly different from that seen with normal grafts, and it was apparently caused largely by the trauma associated with the grafting procedures, since similar pictures are obtained with normal tissues. Leukocytes and macrophages accumulated in and around the grafts, as they do in normal tissues, until the effects of the trauma subsided.

At 4 days (Fig. 11), the white cell invasion had largely subsided. By this time a normal graft would have been well invaded by vessels, but in the tumor there was no vascular growth. As could be seen with the compound microscope, but cannot in the photomicrograph (Fig. 11), the entire observation area of the chamber was covered with a thin layer of tumor cells that formed a clear sheet over the vessels. No evidence was obtained indicating that the cells had invaded the underlying vascular layer at this stage.

Six days after grafting (Figs. 7 and 12), the vessels in contact with the tumor layer showed definite changes, as compared to vessels in a chamber without tumor (Fig. 6). The changes were chiefly in the smaller veins and venous capillaries. They were dilated, and more of them were filled with blood at any given time than would probably have otherwise been the case, thus giving the appearance of increased vessel formation. However, since no sprouting of capillaries was observed and since the apparent increase in number of vessels occurred within about 2 days, a time too short for a fully circulating plexus of such dimensions to form, the effect was almost certainly due to change in already existing vessels rather than to new formation of vessels.

At 8 days (Fig. 13) the changes in veins were more pronounced, and the thickness of the overlying tumor layer had increased until the vessels could not be brought into sharp focus. As far as could be determined, there was no change in the arteries.

At 9 days the dilatation and tortuosity of veins was greatly increased, and the tumor was so thick that photographs showing vessels could no longer be made effectively from the inside of the ear (Fig. 14).

After 9 days, to secure photographs that would show vessels clearly, the ear was turned over and the vascular layer studied from the back, the side opposite to that on which the grafts were placed. Figure 15, taken at the 10-day stage, shows extensive changes in the vessels as compared with the plexus in the chamber before grafting. All veins

and venous capillaries were dilated, and the flow in them was very rapid.

At 16 days (Fig. 16), the larger veins were much larger, and the flow was diminished. Blood oscillated in many of the wide, irregular vascular channels, as though pressure conditions in various parts of the plexus were varying rapidly. At this stage, the first evidence of invasion of the vascular layer by tumor was obtained. Very clear areas developed between some of the vessels which, subsequent studies indicated, were caused by masses of tumor. From this time on, replacement of the vascular layer by tumor was rapid and by 29 days nearly complete (Fig. 20). The vessels appeared to be eliminated by external pressure upon them (Figs. 17–19). There was no rupture of vessels or extravasation of blood.

Histological sections of fixed material taken from the margin of leg tumors and others taken from the edge of ear tumors before they began regression were similar in structure. Blood vessels were in both cases very wide, irregular in calibre, thin-walled, and filled with blood. The situation, as far as the vessels were concerned, appeared to be the same as that observed in chambers at the 22-day stage (Fig. 18).

REMARKS ON THE VASCULARIZATION OF GRAFTS FROM NORMAL TISSUE

The final pattern of vascularization in autografts of normal tissue generally resembled that of the whole organ from which the grafts were taken; in the case of spleen (11), for example, the vasculature of established grafts contained penicillus arteries, ampulae of Thoma, sinusoids, and other structures characteristic of whole spleen. The vascular invasion of normal tissue began within 24 hours, and the definitive pattern for each tissue became established within about 4 days. Vascularization began by sprouting from the vessels on which the grafts were placed. It was not established that portions of the original vasculature included in the graft played any part in the process. An intricate capillary plexus formed rapidly within the graft. Individual capillaries in this plexus were wider than older ones. Within this indifferent plexus, arteries and veins differentiated, and, as they did, the diameter of the intervening capillaries diminished. The process was, in general, the same as that occurring during development of the embryo and was described many years ago by Clark (2) and others.

Grafts of normal tissue in chambers did not increase much in size. An exception to this was spleen. Grafts of this organ might triple in size but thereafter increased no more, even after removal of the spleen. After vascularization was established, further growth of the graft was not accompanied by ingrowth of new capillaries from the host tissues. Further vascularization appeared rather to be an extension of the established vascular system. In grafts, as in whole organs or parts, the vasculature was not a fixed set of channels but, except for the largest veins and arteries, underwent constant but generally very slow changes in size, arrangement, and number of finer channels.

The case was somewhat different for epidermis. This tissue, when implanted in a chamber, tended to become walled-off by connective tissue. It persisted for weeks as a sort of pearl and eventually disappeared. However, with plastic or glass chambers, epidermis occasionally grew into the observation space from adjacent intact skin. When it did, either it was walled-off and eventually disappeared or it continued to grow. In the latter case, it ruined the chamber. It generally occurred as a thick line of cells, very white and glistening, that extended across a portion of the observation space. Capillaries did not invade the epidermis, and vessels were present on only one side of the line of cells. These vessels underwent alteration, becoming much dilated and even sinusoidal in nature. If the epidermis was not walled-off, the end-result of this change was that all vessels disappeared from the observation space and the epidermis of one side of the ear joined that of the opposite side, thus epithelializing all surfaces and causing extrusion of the chamber. Large marginal veins were common around some other types of grafts of normal tissue, but the type of wide sinusoidal vessels found near an advancing line of epidermis in a chamber appeared to be distinctive for that tissue, not having been found in relation to any other tissues studied in grafts with chamber methods. This type of vasculature was temporary. The end-result, as previously stated, was either that the epidermis disappeared and the chamber vasculature was restored to approximately its original condition or that the growing epidermis gained the ascendancy, the vessels disappeared, and the epidermis of one side of the ear met that of the opposite—thus leaving a hole in the ear with its margins epithelialized, the hole being that into which the chamber was introduced.

In chambers of the type used, epidermis did not, as one might expect, form a covering layer over the vessels such as happens in the healing of a superficial wound. This seemed to be related to pressure conditions and to the confined and limited space available for growth.

The behavior of epidermis in chambers has been extensively studied by Clark and Clark (3). Some of their conclusions are:

"The extension, by cell migration, of a line of new epidermis is slightly more rapid than the extension of a growing line of fibroblasts and blood capillaries."

"Fibrin that is near newly differentiating epidermis dissolves (fibrinolytic enzyme)."

"The blood vessels do not penetrate newly formed epidermis. The nearest vessels are always wide with extremely active circulation."

"The connective tissue which forms next to the epidermis is coarse and arranged in parallel rows in contrast with the finer irregularly arranged connective tissue fibers present elsewhere. This probably indicated a genetic difference between dermal and subdermal tissue."

DISCUSSION

The studies of Ide, Baker, and Warren (5), and of Algire and Chalkley (1) indicate that certain malignant tumors have the ability to stimulate the growth of vessels, and the latter authors suggested that the ability of malignant cells to elicit continued growth of new capillaries may be an important expression of neoplastic change. Such a generalization would not seem to apply to all malignant growths, since, from the present studies, it is clear that the V2 carcinoma has practically no ability to stimulate the growth of endothelium. The tumor would seem to have depended for its nutrition upon vessels present in the region being invaded. However, upon these vessels it had a great effect, which was manifested by venous and capillary dilatation without obvious arterial change. A hyperemia was then produced. Coman and Sheldon (4) stated that "the first visible effect of an implanted tumor upon the blood vessels of the host is local hyperemia." They also concluded that "the hyperemia around transplanted mouse tumors is due to the presence of proliferating cells," and not to the malignant nature of the cells. In their opinion, "hyperemia develops because the tumor grows, not vice versa." According to that concept, it might be supposed that in the present instance proliferating cells produced some substance that acted directly on the capillaries and veins but not in a way that caused proliferation of endothelium or that it acted upon the ground substance or that both factors were involved. If the ground substance were rather suddenly softened, then the peripheral vascular support would be reduced, and the veins and capillaries could dilate. Once the vessels were dilated, the tissue between them was invaded by growing tumor cells. As growth continued, it resulted in what appeared to be mechanical compression of the vessels until they disappeared and the area became necrotic. Extension of the tumor depended partly on the availability of already established vessels present in the region being invaded and not at all on the tumor's ability to stimulate its own vascularity by the new formation of capillaries.

The vasculature beneath the epidermis of whole skin is not characterized by wide, sinusoidal vessels, as is the case beneath epidermis that has invaded the limited space in a chamber. Therefore, the vascular response induced by epidermis in chambers must be regarded as part of a healing process which has as its end-result the extrusion of a foreign body and subsequent epithelialization of a free surface, with restoration of the vascular conditions found in normal skin. This indicates that normal epidermis in the presence of injury or under conditions such as those found in chambers is capable of producing something that affects the vessels as described, and this it does not do when acting as the intact covering layer of the skin. The V2 carcinoma, a tumor of epidermal origin, also seemed to produce something that caused vascular dilatation in a manner very similar to that in which normal epidermis affected vessels under the conditions stated above. If so, then to that extent the tumor retained and continually used a capacity of its normal cellular ancestors which the latter use only occasionally—that is, when attempting to extrude a foreign body or, perhaps, to repair an injury. A further similarity between the tumor and epidermis, as seen in chambers, was that neither tissue was invaded by blood vessels. All normal tissues except epidermis, thus far studied in this laboratory, were initially invaded by vessels, whether they survived for long periods in grafts or not. The V2 carcinoma also behaved in that respect as its normal cellular precursors.

It would seem significant that, when the V2 carcinoma was implanted into leg muscles, the result was death of the animal, but when implanted in the ear the tumors all underwent regression ending in complete healing of the ulcerations, with no deaths. Since leg tumors were much larger than the combined ear tumors, it may be suspected that death was related to volume of tumor. As to what caused death no certain evidence was obtained, but it was noticed that after tumors reached a certain size the animals no longer ate, and, since death was preceded by great loss of weight, it is possible that starvation may have been a factor.

As to why subcutaneous ear tumors regressed, it is felt that the nature of the tissue in the ear which was invaded by the tumor and the restricted space and lower temperature in the ear were probably not important factors. Tumors in the

ear grew rapidly for a time, and, from the standpoint of the mechanics of the region they were in, there would seem to be no reason why they could not have extended until the whole ear was involved. The histology of the edge of an advancing ear tumor and of a tumor in muscle were the same, as far as could be determined. One important difference between the two cases was that all ear tumors were infected secondarily. The significance of this is being investigated.

In one chamber there were spicules of bone present before the tumor grafts were made. The bone rapidly disappeared as the tumor extended. Either the tumor produced a toxin or some other material that destroyed the bone or it disappeared as the result of pressure exerted by the growing cells. Since there was no more sticking of leukocytes to vessel walls than occurs from time to time in grafts of normal tissue, it is supposed that the tumor was not producing a toxin, unless it produced one that was specific for bone. The bone disappeared long before blood flow was seriously impaired in the chamber. It is well known that pressure constantly applied to bone causes it to be resorbed. That pressures in chambers were increased by multiplying tumor cells is clear from the vascular responses. This pressure increase could have been sufficient to cause disappearance of the bone.

SUMMARY AND CONCLUSIONS

The V2 carcinoma of Kidd and Rous survived readily when implanted subcutaneously into rabbits' ears. The tumors ulcerated, and after about 6 months underwent regression with complete disappearance and epithelialization of the ulcerated surfaces. These tumors were all secondarily infected. When implanted into muscles, the tumors killed the animals in from 6 to 10 weeks.

When implanted into transparent chambers installed in rabbits' ears, the tumor grafts survived and completely obliterated all vessels in the chambers in about 30 days. Vascular response to the tumor in grafts bore no relationship to that of any of the 12 types of normal tissue used for comparison except for epidermis, the tissue from which the tumor was derived. It behaved like normal epidermis, responding to injury to the extent that it produced dilatation of the host vessels and was not invaded by capillaries.

The V2 carcinoma had no ability to stimulate the growth of vessels but depended for its nutri-

tion, as far as vessels were concerned, on the vasculature already present in the region being invaded.

The multiplying tumor cells in chambers appeared to destroy the vessels on which they had been placed, and, hence, their only vascular supply, chiefly by external pressure upon the vessels. The result was that the tumors had necrotic centers. The only part of the tumor that survived was a narrow rim at the periphery where there were vessels of the host region not yet completely affected by growth pressures or other factors in the tumor.

The ability to elicit continued growth of vascular endothelium is not a characteristic of all neoplasia, since the V2 carcinoma, under the conditions given, does not have that property at all.

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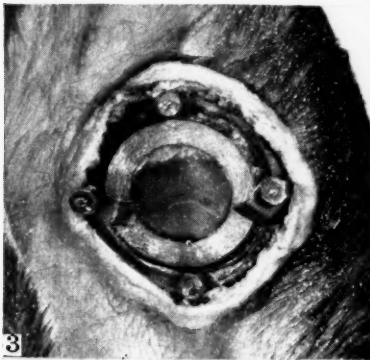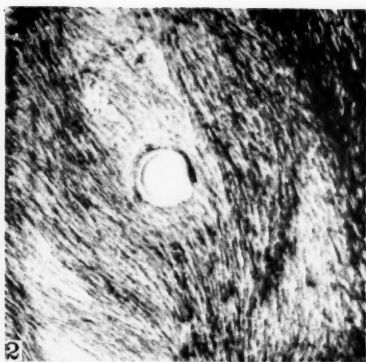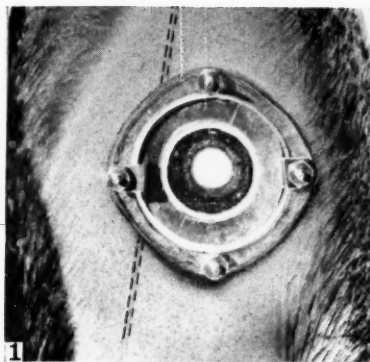
Figs. 1 and 2.—A tantalum and mica chamber installed in an ear. Figure 1 was taken from the inside and 2 from the outside. The dotted line represents the position of the central artery. The observation space is the central clear circle. It is filled with a thin layer of vessels, not visible in the photographs, which can be exposed and on which grafts may be placed.

Figs. 3 and 4.—Similar photographs of another chamber in which two grafts of V2 carcinoma were placed 9 weeks previously. The raised rim around the chamber in Figure 3 is connective tissue and not associated in any way with the tumor. The tumor obliterated all vessels in the observation space, extended through the ear, and produced the mass shown in Figure 4.

Fig. 5.—Tumors 1 month after implantation of grafts of V2 carcinoma in subcutaneous tunnels between outside skin and cartilage.

Fig. 6.—The vasculature in a chamber without grafts 6 months after installation. ×16.

Fig. 7.—The vasculature in a chamber in which two grafts of V2 carcinoma had been placed on the vessels 6 days previously. Tumor cells grew as a thin sheet, not visible in the photograph, over the vessels and produced the venous and capillary dilatation shown. ×16.

Fig. 8.—Two subcutaneous tumor grafts 8 weeks after placing, illustrating ulceration of the center which occurred eventually in all subcutaneous ear grafts. After about 6 months these tumors disappeared and the ulcerated surfaces were epithelialized.

Figs. 9–20.—Photographs of the same chamber at various intervals. ×16.

Fig. 9.—The vessels immediately after placing two grafts of V2 carcinoma. Unlike grafts of normal tissue, the nature of the tumor was such that the grafts are not clearly visible. They lie one on each side of the tortuous vein just above center. X is a spicule of bone present in the chamber before the grafts were made.

Fig. 10.—Two days later.

Fig. 11.—Four days later. The effects of trauma associated with the grafting operation have largely subsided. Tumor cells cover the area as a thin sheet.

Fig. 12.—Six days after grafting. The vascular changes are similar to those shown in Figure 7.

Fig. 13.—Eight days after grafting, dilatation of vessels was greater.

Fig. 14.—At 9 days, the tumor layer was so thick that vessels could not be brought into sharp focus; therefore, the ear was turned over and all subsequent pictures were made from the side of the chamber opposite to that on which the grafts were placed.

Fig. 15.—Ten days after grafting.

Fig. 16.—Sixteen days after grafting. The clear areas near the bottom of the photograph are places where the tumor first invaded the vascular layer.

Fig. 17.—Twenty-one days after grafting. Pressure effects on the vessels are evident.

Fig. 18.—Twenty-two days after grafting, pressure effects are marked.

Fig. 19.—Twenty-seven days after grafting, many vessels have disappeared.

Fig. 20.—Twenty-nine days after grafting, the vasculature in the chamber has been largely eliminated.
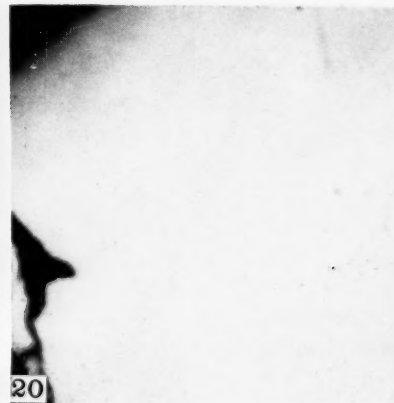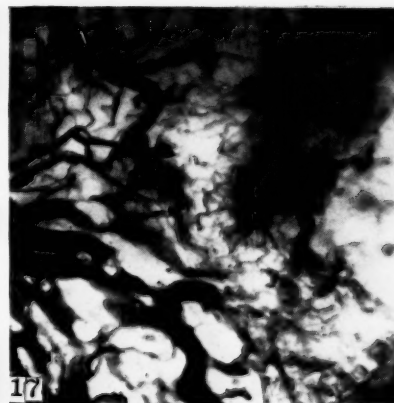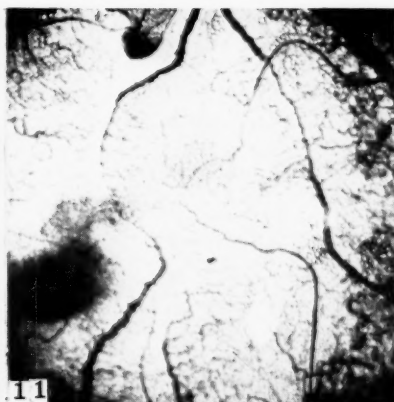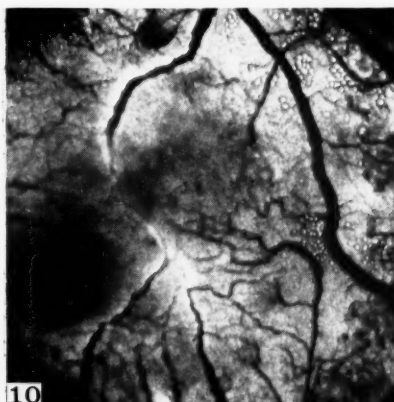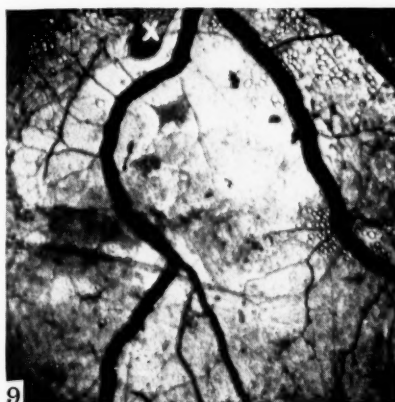
Observations on Inhibition of Nucleic Acid Synthesis Resulting from Administration of Nitrogen Mustard, Urethan, Colchicine, 2,6-Diaminopurine, 8-Azaguanine, Potassium Arsenite, and Cortisone*

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It has long puzzled those interested in the chemotherapy of cancer why such diverse agents as are mentioned in the title of this paper should provide temporary palliative effects in certain types of neoplastic disease, while hundreds of other compounds, some closely related structurally, should be without effect. Most of the known anti-cancer agents which have been studied extensively have been shown to be carcinogenic and under certain experimental conditions cause nuclear damage, inhibition of mitosis, chromosome breaks, and the production of mutations (1). This knowledge would suggest that one underlying similarity in the heterogeneous list of anti-cancer agents might have to do, directly or indirectly, with chromosome metabolism.

It is well established that the chromosome is composed largely of desoxyribonucleoprotein (10).

The fact that two well-known purine antagonists in bacteria, 2,6-diaminopurine and 8-azaguanine, will in certain instances preferentially inhibit neoplastic cell growth (3, 9) is strong evidence that the mechanism of these compounds at least has to do with nucleic acid metabolism. Our recent observations that 8-azaguanine, as such, is apparently fixed in mouse nucleic acids (11) and that folic acid antagonists inhibit purine synthesis (16) are further evidence implicating the nucleoprotein system in temporarily effective cancer chemotherapy. It has also been reported that x-radiation significantly depresses nucleic acid synthesis (7, 15).

In view of the above-mentioned facts, it was considered of interest to study the effect of a series of known anti-cancer agents on the *in vivo* synthesis of nucleic acids and fractions thereof.

The formation of the carbon chain of uric acid, therefore, presumably also of the purines of nucleic acid, has been studied with the aid of carbon 13 (2, 17). It was concluded that the sources of the component atoms of the purine skeleton were as follows:
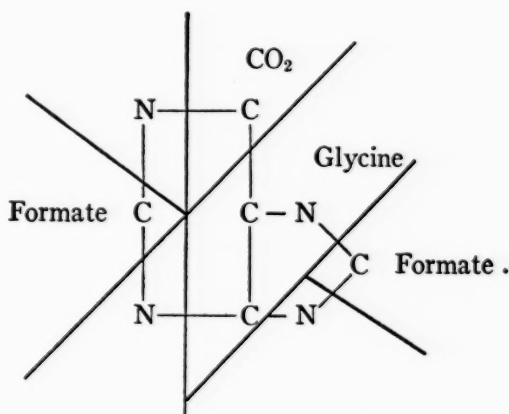
Totter *et al.* (18) have recently shown that, in the rat, formate carbon is indeed a precursor of desoxyribonucleic acid guanine, adenine, and thymine (methyl group) and ribonucleic acid guanine and adenine.

It is theoretically possible, with the use of a labeled precursor of the nucleic acid purines, to determine the rate of synthesis of these genetically important compounds. Also, once a base line for the incorporation of the labeled precursor is established, one should be able to determine the effect of various agents on the synthesis of nucleic acid purines in the intact animal. Attempts to determine the effect of nitrogen mustard (methyl bis-

[2-chloroethyl]amine), urethan, colchicine, 2,6-diaminopurine, 8-azaguanine, potassium arsenite, and cortisone on nucleic acid synthesis have been made and are reported herein.

EXPERIMENTAL

Isolation procedure.—Carbon 14-labeled sodium formate (1.4 μc.) was injected intraperitoneally into groups of four adult CFW strain mice. After 6 hours these mice were sacrificed, and the viscera (liver, spleen, washed intestines, kidneys, and testes) were mixed with an equal weight of viscera from noninjected mice in order to provide sufficient starting material for isolation of fractions of particular interest. This mixture of tissues was then homogenized in the cold, with a jacketed Waring Blendor. Aliquots of the homogenate were assayed for radioactivity. The viscera homogenate was dehydrated with several portions of absolute ethanol and ethyl ether. The dehydrated tissue was extracted with 10 per cent NaCl and the crude sodium salts of the nucleic acids precipitated with alcohol and redissolved in water; the nucleic acids were precipitated with HCl and alcohol, washed with water, and dried with alcohol and ether (18). To obtain desoxyribonucleic acid (DNA), a portion of the combined nucleic acids was twice hydrolyzed for about 18 hours at room temperature in 2 N sodium hydroxide and reprecipitated in acid alcohol (13). Another portion of the combined nucleic acids was hydrolyzed in 0.5 N HCl for 1 hour and the purines precipitated in acid solution with silver nitrate. These purines were redissolved in 0.5 N HCl and reprecipitated with silver in acid solution.

Examination of isolation procedures.—In order to assure ourselves of the adequacy of the isolation technics for these exploratory experiments, several studies were carried out. A large group of leukemic mice was injected with 1.4 μc. each of sodium formate, and after 6 hours the viscera nucleic acids and nucleic acid purines were isolated and carried through repeated purifications. The results of this investigation are presented in Table 1. Representative samples of the crude twice-precipitated combined nucleic acids used herein were analyzed by the orcinol and diphenylamine color reactions and shown to be about 90 per cent nucleic acids (40 per cent RNA and 50+ per cent DNA). The twice-purified DNA was found to contain 90–95 per cent DNA and no more than 5 per cent RNA by these somewhat inadequate colorimetric analyses.

Reichard has reported that, on acid precipitation of silver purines, precipitation of part of the pyrimidines ("in the form of thymic acid") takes place

(12). We have carried out phosphorus determinations on purines (extracted with acid from silver purines) after 1, 2, 4, and 6 precipitations with silver nitrate in acid solution. The results of these analyses are given in Table 2. An HCl extract from the twice-precipitated purines has also been acid-hydrolyzed in a sealed tube for 2 hours at 180° C. (in order to free pyrimidines, if any) and chromatographed on paper with a solvent system

TABLE 1

THE EFFECT OF REPEATED PURIFICATION ON THE SPECIFIC ACTIVITY OF NUCLEIC ACIDS AND SILVER PURINES FROM FORMATE-INJECTED MICE

No. of purifications	SPECIFIC ACTIVITY (μc/mole C)		
	Combined nucleic acids*	DNA†	Ag purines from combined NA‡
1	61.0	67.6	184.2
2	46.6	51.0	
3	56.4	50.4§	
4		55.0	204.0
6		51.1	200.0

* Repeated purification through sodium salt.

† Repeated treatment with 2 N NaOH (for 18 hours) and precipitation with acid.

‡ Repeated extraction with 0.5 N HCl and precipitation with silver nitrate.

§ This sample is an aliquot of the twice-purified DNA, which was carried through the lanthanum salt, as has been suggested by Hammarsten (6).

NOTE: These isolations were carried out on the viscera from 50 leukemic Akm mice (6 days after inoculation of Ak4 leukemia).

TABLE 2

PHOSPHORUS CONTENT OF SILVER PURINES AFTER REPEATED PRECIPITATION IN ACID SOLUTION

No. of precipitations	Per cent phosphorus
1	0.47
2	0.17
4	0.14
6	0.15

containing 4 parts butanol, 1 part diethylene glycol, and 1 part water in an ammonia atmosphere. This chromatogram showed a barely discernible trace of thymine but no evidence of other pyrimidines.

Injections with anti-cancer agents.—In the experimental groups, mice were treated with a single dose (usually the LD_{50}) of a given anti-cancer agent, or six doses (maximum tolerated level) on successive days followed by an LD_{50} dose. The last injection of the anti-cancer agent was followed immediately by an injection of $HC^{14}OONa$. These experiments were all of 6 hours' duration, and isolation of nucleic acids and purines was carried out in the same manner as has been described above.

TABLE 3

THE EFFECTS OF CERTAIN KNOWN ANTI-CANCER AGENTS ON INCORPORATION OF C[14] (FROM FORMATE) INTO VISCERA, NUCLEIC ACIDS, AND NUCLEIC ACID PURINES

Exp. No.	Treatment	Dosage (mg/kg)	Viscera homogenate	Combined nucleic acids	Combined nucleic acid purines	Ratio† of specific activities of viscera to purines (×10²)
				Specific Activity*		
1	Controls			57.6	138.4	
2	Controls		4.4	71.8	176.8	
3	Controls		4.4	42.4	187.2	
4	Controls		5.2			
5	Controls		4.4			
		(Average)	4.6	57.3	167.5	2.7
6	Nitrogen mustard	4.0	6.6	57.2		
7	Nitrogen mustard	0.75(6×)‡				
		4.0 (1×)	6.8	39.8	135.2	5.0
8	Urethan	1,800.0	7.4	45.4	148.2	5.0
9	Urethan	450.0 (6×)				
		1,800.0 (1×)	8.4	19.8	132.8	6.3
10	Urethan+nitrogen mustard	225+0.5 (6×)				
		900+2.0 (1×)	5.6	23.3	74.8	7.6
11	Benzene	250.0 (6×)				
		1,150.0 (1×)	5.0	38.3	161.0	3.1
12	KAsO₃	4.5 (6×)				
		9.0 (1×)	4.2	22.5	79.8	5.3
13	Colchicine	2.4	6.6	52.4	172.4	3.8
14	Colchicine	0.63(6×)				
		2.4 (1×)	5.5	39.3	163.5	3.4
15	2,6-Diaminopurine	100.0 (7×)	5.8	53.2	98.6	5.8
16	2,6-Diaminopurine	100.0 (6×)				
		248.0 (1×)	7.0	18.5	65.6	10.7
17	8-Azaguanine	31.3 (6×)				
		250.0 (1×)	3.7	27.2	99.2	5.0
18	Cortisone	44.0	4.4	36.7	88.0	5.0
19	Cortisone	44.0	6.3	49.0	133.5	4.7
20	Cortisone	44.0 (3×)	5.3	31.9	98.0	5.4

* Specific activities in μc/mole of carbon.
† Ratio of the specific activities of the viscera homogenate to the combined nucleic acid purines isolated therefrom.
‡ Number of injections indicated in parentheses under dosage.

TABLE 4

THE EFFECT OF CERTAIN KNOWN ANTI-CANCER AGENTS ON OVER-ALL INCORPORATION OF C[14] FROM NaHC[14]O₃ INTO VISCERA

Exp. No.	Treatment	Dosage (mg/kg)	Specific activity of viscera homogenate (μc/mole of carbon)
21	Controls		0.68
22	Controls		0.77
23	Controls		0.90
24	Controls		0.71
25	Controls		0.98
26	Controls		0.99
		(Av.)	0.84
27	Urethan	1,800.0	1.07
28	Urethan	1,800.0	2.04
29	Urethan	1,800.0	2.07
30	Nitrogen mustard	3.0	1.08
31	Nitrogen mustard	4.0	1.18
32	2,6-Diaminopurine	100.0 (7×)	1.15
33	Benzene	1,150.0	1.22
34	Potassium arsenite	18.0	0.62
35	Colchicine	2.4	1.38

NOTE: All treatment was on the basis of a single intraperitoneal injection except 2,6-diaminopurine, which was given at the level of 100 mg/kg daily for a total of seven injections.

TABLE 5

INCORPORATION OF C[14] FROM BICARBONATE IN NUCLEIC ACIDS AND NUCLEIC ACID PURINES OF CONTROL MICE AT 6 HOURS

Exp. No.	Combined nucleic acids	DNA	Combined nucleic acid purines	DNA purines
		Specific activity (μc/mole C)		
21				1.41
22				1.76
23	1.65	1.95		2.10
24	2.32	2.22		
25	2.50		3.27	
26		2.63		
(Av.)	2.16	2.26		1.76

NOTE: All these experiments have been carried out on pooled viscera from four mice injected with 1.4 μc. of sodium formate each.

TABLE 6

TURNOVER OF C[14] FROM FORMATE IN NUCLEIC ACIDS AND NUCLEIC ACID PURINES FROM MICE

	1 hour	6 hours	24 hours
	Specific activity (μc/mole C)		
Viscera homogenate	3.9	4.6	3.8
Combined nucleic acids	36.7	57.3	31.7
Combined NA purines	77.2	167.5	75.8

NOTE: The 1-hour and 24-hour experiments represent pooled tissues from four mice each, while the 6-hour experiment is average data involving 12–16 animals. The dosage of formate has been uniformly 1.4 μc. per mouse.

Similar studies have been carried out using $C^{14}O_2$, a less specific precursor of the 6-carbon atom of the purine skeleton. In these experiments $NaHC^{14}O_3$ was injected at a level of 11.8 $\mu c.$ per mouse, and viscera were assayed for carbon 14 content.

The dosages employed and the comparative specific activities of viscera homogenate, combined nucleic acids, desoxyribonucleic acid (when isolated), and nucleic acid purines at 6 hours after injecting active formate and carbonate are presented in Tables 3, 4, and 5.

We have observed that there is little free radioactive formate or bicarbonate carbon in mouse tissues at 6 hours after injection. In order to obtain some information on the rate of turnover of formate carbon in nucleic acids and nucleic acid purines, experiments were carried out in which activities were determined at 1, 6, and 24 hours after injection (Table 6).

All carbon 14 determinations were carried out by a gas phase procedure, which has been described (14). The specific activities reported have been corrected for the original dilution of active viscera with inactive viscera.

DISCUSSION

From data presented in Tables 1 and 2 and from results mentioned in the experimental section, it appears that the twice-purified DNA and the nucleic acid purines are of a purity adequate for the objectives of these exploratory experiments. Less reliance can be placed on the absolute accuracy of the combined nucleic acid values.

In Tables 3 and 4 it can be seen that, in general, nitrogen mustard, urethan, colchicine, and 2,6-diaminopurine have caused an increase in the over-all incorporation of formate and bicarbonate carbon into the visceral organs at 6 hours. Hevesy has reported a similar increase in tissue incorporation of carbon 14 from carboxyl-labeled acetate following the injection of urethan or the administration of x-radiation (8). Hevesy interpreted this enhanced carbon 14 fixation, observed on administration of urethan, to be due to a decreased catabolism, which, in turn, slowed down the decrease in specific activity of carbon dioxide and acetate with time (dilution with normal catabolic products) and thus promoted incorporation of carbon 14 into tissue constituents. If such an interpretation were adapted to the present data, it would appear that the above-mentioned anti-cancer agents have depressed metabolic processes responsible for production of CO_2 and formate in the animal.

From data summarized in Table 3, it can be seen that in addition to folic acid antagonists (16) and x-radiation(7, 15), which have previously been reported to possess inhibiting action on nucleic acid synthesis, 2,6-diaminopurine, 8-azaguanine, cortisone, potassium arsenite, and the combination of nitrogen mustard plus urethan all markedly reduced incorporation of formate into nucleic acid purines. If one considers the ratios of the specific activities of the visceral organs to the nucleic acid purines from mice treated with nitrogen mustard or urethan alone, a very significant alteration can be seen. After administration of urethan or nitrogen mustard, viscera specific activities at 6 hours after injection of $HC^{14}OONa$ have increased by 40–80 per cent over the control groups, while the nucleic acid specific activities are slightly lower than those of the control animals. Benzene and colchicine have been relatively ineffective as nucleic acid inhibitors. All the present comparisons were necessarily made at levels which take into consideration the relative toxicities of the chemotherapeutic agents.

The inhibition of nucleic acid synthesis observed in certain of the present experiments does not necessarily indicate that the site of action of a given agent is directly on some moiety of the chromosome or nucleoprotein molecule, although this may be the case with the purine antagonists (11). There is the obvious possibility that the inhibition observed might be directed toward the source of energy for nucleic acid anabolism, on some precursor or precursor system, or perhaps more likely on enzyme systems having to do with nucleic acid metabolism. In a single experiment with four mice, we have observed that starvation (3 days) resulted in an apparent increase in over-all tissue fixation of formate in 6 hours and, at the same time, a reduction of the incorporation of carbon 14 from this source in nucleic acids and nucleic acid purines to about half that observed with control mice.

It is of considerable interest that CO_2 fixation in the combined viscera nucleic acids is of the same order as in desoxyribonucleic acid or in the nucleic acid purines (Table 5). This fits in well with the pattern of observations that purine precursors are incorporated into DNA and RNA at rates which are of the same order (4, 18). In contrast, adenine has been shown to be incorporated into RNA at a much higher rate than into DNA (5). In the case of radioactive formate-injected mice, the nucleic acid purine specific activities have usually been 3–4 times that of the crude combined nucleic acids.

As shown by the results given in Table 1, as compared to control animals in Table 3 and much

unreported data, we have observed that leukemic mice fix significantly more formate carbon in viscera nucleic acids than do control mice at 6 hours.

Studies are now under way with more refined isolation procedures (ion-exchange chromatography) to determine the effect of known anti-cancer agents and certain hormones on the synthesis of DNA guanine, adenine, and thymine and RNA guanine and adenine.

SUMMARY

Using the technic of measuring the incorporation of formate carbon into nucleic acid purines of control and treated mice, data have been obtained which indicate that 2,6-diaminopurine, 8-azaguanine, cortisone, potassium arsenite, urethan, and nitrogen mustard inhibit nucleic acid synthesis *in vivo*. Benzene and colchicine have been relatively ineffective as inhibitors of nucleic acid synthesis under the conditions of these experiments.

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Book Reviews

Amino Acids and Proteins. COLD SPRING HARBOR SYM-POSIA ON QUANTITATIVE BIOLOGY, Vol. 14. New York: The Biological Laboratory, Cold Spring Harbor, 1950. Pp. 217. $7.00.

The selection of *Amino Acids and Proteins* as the topic for the fourteenth Cold Spring Harbor Symposium reflects the rapid progress that has been made in this field since the sixth (1938) symposium which dealt with "Protein Chemistry." It is the only subject to which a second Cold Spring Harbor Symposium has been devoted. This position aptly emphasizes the major role that the proteins play in biology.

The fourteenth volume of this excellent series maintains the high standard of quality established by its predecessors. In the foreword it is mentioned that it was anticipated that there would be some duplication of the sixth symposium, but the reviewer has found little overlapping. There are 22 papers presented in Vol. 14, and of the 33 authors, only 3 had papers in Vol. 6. It is impossible to review all the articles adequately; however, it seems desirable to mention names and general titles.

The first paper by H. B. Bull deals with the molecular weight distribution of peptides in protein hydrolyzates. J. R. Cann and J. G. Kirkwood discuss results obtained in their application of a new promising technic for "The Fractionation of Proteins by Electrophoresis-Convection." The success that has been realized in separating amino acids and polypeptides by countercurrent distribution is described by L. C. Craig, J. D. Gregory, and G. T. Barry. Nearly one-third of the papers deal with some aspect of the amino acid composition of proteins. W. H. Stein and S. Moore present data on the "Chromatographic Determination of the Amino Acid Composition of Proteins." Two papers deal with applications of the isotopic derivative technic to the analysis of proteins. The first, by A. S. Keston and S. Undenfriend, discusses its general application, and the second, by M. Levy and E. Slobodiansky, describes the application to silk fibroin. F. Sanger summarizes his interesting results on "Some Chemical Investigations on the Structure of Insulin," from which it is concluded that the insulin molecule is built up of four open polypeptide chains. The quantitative analysis of amino acids in insulin and lysozyme is discussed by C. Fromageot. In a stimulating paper on "Structure and Enzymatic Breakdown of Proteins," K. Linderstrom-Lang discusses the role of denaturation in the enzymatic hydrolysis of proteins. L. R. M. Synge reviews his own and other work dealing with "Physical and Chemical Studies of Gramicidin and Some Implications for the Study of Proteins." A paper on "X-Ray Analysis and Protein Structure" by Dorothy Crowfoot Hodgkin gives a good summary of the x-ray data that have been obtained on very large proteins as well as smaller proteins and peptides, with a stimulating discussion of the structural interpretations. Under the title "Size Relationship among Similar Proteins," K. O. Pedersen discusses variations in the sedimentation constants of different preparations of similar proteins and the association and dissociation reaction of protein units. The results suggest that the composition of the same protein from different individuals may vary within limits.

Two papers deal with the cytochemistry of proteins. J. F. Danielli describes the use of chromogenic reagents along with specific blocking reagents, and B. P. Kaufman, H. Gay, and M. R. McDonald describe the use of enzymes for the localization of cellular proteins. Two papers by S. Emerson and D. Shemin deal with different aspects of the biosynthesis of amino acids. "The Metabolism of Peptides" is discussed by J. S. Fruton and S. Simmonds. I. M. Klotz describes "The Nature of Some Ion-Protein Complexes." In a very interesting paper entitled "Some Considerations of the Interaction of the Metal Peptidases with Their Substrates," E. Smith and R. Lumry discuss the forces that are involved in enzyme substrate complex formation and the factors affecting peptide hydrolysis. Two papers should be of special interest to those who are concerned with cancer research. In one of those, J. M. Luck describes studies on "The Liver Proteins" and the effect of carcinogens on liver components. The last paper by P. C. Zamecnik and I. D. Frantz is concerned with radioactive tracer experiments on "Peptide Bond Synthesis in Normal and Malignant Tissues."

Many of the papers present information which as yet is not found elsewhere, others give conclusions already published in detail. The discussion that follows each paper and the freedom of expression that the symposium offered give Volume 14 an added value.

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Metabolism and Function. D. NACHMANSOHN, ED. New York: Elsevier Publishers, 1950. Pp. 348. $7.00.

This book is a collection of 38 scientific papers, published originally as Vol. 4, pp. 1–348, of *Biochimica et Biophysica Acta*, in honor of Otto Meyerhof on the occasion of his 65th birthday.

There are 28 papers in English, 5 in German, and 5 in French. With one or two exceptions, all papers include adequate summaries in all three languages. Adequate bibliographies are also given. Six photographs, including

a large portrait of O. Meyerhof, and group pictures of Meyerhof and his colleagues taken in 1928, 1931, and 1949 add interest to the book.

There is a 2-page introduction by D. Nachmansohn which traces all too briefly Meyerhof's main scientific achievements. A more comprehensive treatment of the historical development of Meyerhof's work, and of the interrelations of his contributions with those of his chief contemporaries, would have served as an integrative framework for the individual papers, and could have made of this volume a more definitive contribution to the science of cellular physiology. This lack is partly filled by the introductions to some of the papers, and especially by the contributions of A. V. Hill and A. von Muralt.

The book is divided into Part I, "Muscle"; Part II, "Nerve"; Part III, "Drug Action"; and Part IV, "Intermediary Metabolism." The editor, in the biblical tradition, has served the good wine first. The section on muscle comprises seven papers, the first of which is a ringing challenge by A. V. Hill for biochemists to return to the study of the living muscle. The six remaining papers, including contributions by leading workers in the modern "myosin" era of muscle physiology, are unanimous in their failure to meet the challenge. These papers are marked by a uniformity of approach, essentially that of the protein chemist, and by a uniformity in the object of study, viz., the contractile protein of the muscle cell. Nevertheless, these papers attest to the extent to which the protein chemists of the "myosin" era are revealing the intimate nature of the mechano-chemical coupling within the muscle cell and suggest that the challenge of A. V. Hill will perhaps be met by an encircling maneuver and not by a frontal attack. These six papers bring one abreast of the latest developments in this field of investigation.

Part II, on "Nerve," is marked by an outstanding 3-page essay by A. von Muralt, in which he draws a lesson, for neurophysiology, from the history of muscle physiology. There are two papers from the Nachmansohn group reporting recent studies on ion permeabilities in relation to nerve function. The combination of radioactive ions and the squid giant fiber have made these studies possible. Papers on nerve and muscle morphology, the response of nerve to azide and fluoroacetate, and a paper on the functional organization of the brain complete this section. The unity of purpose so characteristic of the papers on muscle, and the lack of such unity in papers on neurophysiology, flows out of the fact, as highlighted by von Muralt, that "The ground for neurophysiology therefore is not as well prepared [today] as it was for muscle physiology in 1907."

The remainder of the book is a heterogeneous collection of 25 papers, ranging from pure chemistry to classical mammalian physiology. An outstanding paper in this last section is that of G. Wald, "Interconversion of the Retinenes and Vitamins." The interconversion of the retinal pigments is reviewed, and evidence for the implication of the classical Meyerhof-Embden glycolytic scheme, via DPN as the coenzyme of retinene reduction, is presented.

Of particular interest to students of the cancer problem is the paper on abnormalities in cell division by E. Boyland, in which the significance of the dual mutagenic and carcinogenic properties of a variety of agents is discussed. A 21-page paper by H. A. Krebs, which may be of use to workers in the cancer field, contains an extensive analysis of suspension media for tissue slice work. There is a paper by H. M. Kalckar on purine and pyrimidine incorporation into nucleic acid.

Most of the remaining papers are concerned with detailed aspects of various problems in intermediary metabolism. There are four papers dealing with aspects of glycolysis and fermentation. Other subjects discussed are: some of the reactions of the Krebs citric acid cycle; acetaldehyde metabolism; acylation reactions; lipase and phosphatase activity; "free" and "bound" forms of the DPN-glyceraldehyde phosphate dehydrogenase. A detailed presentation by O. Warburg and co-workers of certain calculations relevant to the controversy over the quantum efficiency of photosynthesis completes the book.

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Form- und Wachstumsgesetze drüsenbildender Karzinome.
By RICHARD BÖHMING. Stuttgart: Georg Thieme Verlag, 1950. Pp. 105.

The starting point of the author's considerations are three classical theories of a general biological nature, namely: (1) Albrecht's doctrine of the "organoid" character of tumor growth, stating that a tumor, benign or malignant, is not a lawless mass of cells but is ruled by definite morphogenetic laws; (2) Heidenhain's theory of histosystems, especially the statement that glandular organs develop by a process of dichotomy, the "adenomeres" thus formed remaining connected to build up tree-like formations of an ever higher order; and (3) Spemann's doctrine of embryonic induction. The author emphasizes that the usual inspection of individual slides leads to the impression that undirected growth is characteristic of tumor. The principal merit of his own work is the careful technic—namely, the use of serial sections in proper orientation and of plastic reconstructions. Based upon these methods, he comes to the conclusion that adenocarcinomas follow the rule of adenomeric growth. The same applies to metastases. Malignant growth is trespassing not the morphological, but only the physiological laws of the organism: in normal glandular tissue, growth and division are limited by the superordinated organization of the organ, while they are unlimited in adenocarcinomas. Because the development of adenocarcinomatic tissues corresponds largely to the development of normal glandular tissue, the general conceptions of developmental mechanics remain applicable such as Spemann's embryonic induction and organizer substances, the mutation theory of cancer, the concept of gene hormones, etc.

The handicap of a merely morphological approach to the tumor problem is, of course, the difficulty of drawing

unequivocal conclusions from the microscopic pictures, especially in the transition stages from normal to hyperplastic and malignant tissue. The interpretation given cannot readily be generalized by other types of carcinomas. The author makes no reference to any recent non-German publications, which is understandable, considering the conditions in postwar Germany. But the book presents many problems which are seldom envisaged in the usual mainly physiological and experimental approach.

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Progress in Biochemistry. By FELIX HAUROWITZ. New York: Interscience Publishers, Inc., 1950. Pp. 405.

In the fifth edition of this series, the first to be written in English, the author has attempted to summarize the most important advances and the current status of the major fields of biochemical investigation. This expansive subject has been divided among 24 chapters, which have been written in a clear and concise manner. In most instances the historical introductions have been omitted for the purpose of conserving space; and, instead, the emphasis has been placed on experimental findings published since 1939. Inherent in the abbreviated approach was the necessity for a certain amount of generalization based on the selection of key papers; this has been accomplished by the author in an accurate and admirable manner which should be of assistance to intermediate students of biochemistry, pharmacy, medicine, and zoölogy.

Of special note are chapters on "Intermolecular Forces in Living Matter," "Thermodynamics and Kinetics in Biochemical Reactions," "Protein Chemistry," and "Protein Metabolism." The latter two chapters have 255 and 179 references, respectively. Other chapters deal with nucleic acids, sterols, protein hormones, fatty acid metabolism, mineral metabolism, enzymes, and isotopes. The subject of vitamins and growth factors has been surveyed very briefly and could be improved considerably by the inclusion of more information of the production of the deficiency states and the relationship of the vitamin to the altered metabolism. The chapter on carbohydrate metabolism deals comprehensively with the assimilation and glycolytic breakdown of hexoses but omits a discussion of its relationship to the tricarboxylic acid cycle. This important subject, however, has been dealt with briefly in the discussion of fatty acid metabolism. Two additional chapters on "Immunochemistry" and "Cytochemistry" are also included and should be of value in orienting the general student of biochemistry. In general the text is well documented.

It is the opinion of the reviewer that this book, although not a penetrating compendium, should be of particular value to intermediate students of biochemistry and medicine, in that it constitutes a concise survey of the recent progress on most biochemical research fronts.

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Chemical Embryology. By JEAN BRACHET. Translated by LESTER G. BARTH. New York: Interscience Publishers, 1950. Pp. 533. $8.00.

The translation by Dr. Barth of the second French edition of Brachet's *Embryologie chimique*, which appeared in 1945, will extend the audience this excellent work has enjoyed for several years. The translation is accurate and very readable.

The emphasis of the book, as the author has stated in his preface to the first edition, is on the study of the egg and the morphogenesis of the embryo, with purely biochemical facts subservient to this purpose. After a preliminary survey of the biochemical and biophysical methods available to the chemical embryologist, the discussion concerns itself with the chemical basis of sex determination, formation of gametes, fertilization, cleavage, the nucleic acids, growth, differentiation, and metabolism. The remaining chapters discuss the chemical embryology of the organization center, and regeneration; a final chapter is devoted to "facts acquired, controversial points, and the future outlook."

Perhaps the most striking characteristic of the work is the presentation of data supporting both sides of controversial questions, followed by the author's expert criticism of faulty thinking or faulty technic where these have occurred.

Since the egg is a specialized type of cell and since many of the metabolic processes of the embryo are cellular processes, this book will be of as great interest to the experimental cytologist as to the reader primarily interested in chemical embryology. And because of the importance of the problems of growth and differentiation to cancer research, it will be of great value to the oncologist.

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